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MENGO VIRUS PROTEINS:
CAPSID STRUCTURE; CLEAVAGES *in vivo*

by

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A THESIS

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To my family

ABSTRACT

The asymmetric structure unit of the Mengo virus capsid consists of one molecule each of the proteins α, β and γ. Structure units are clustered in pentamers which are centered at each of the 12 vertices of an icosahedral (T = 1) lattice. The location of the 60 δ polypeptides in the capsid is not known. In order to obtain information about the arrangement of the α, β and γ polypeptides in the structure unit and the location(s) of the δ polypeptides in the capsid, virions have been treated with the bifunctional cross-linking agents dimethylsuberimidate (DMS), dimethyladipimate (DMA) and dithiobis(succinimidyl propionate) (DSP), and gel electrophoresis procedures have been used to detect the polypeptide complexes produced. Positive identification has been made of α_2, α_3 and αγ complexes crosslinked with DMS. With DSP, positive identification has been made of βγ, αβ, αβγ, α_3 and α_4 complexes. None of the chemical crosslinking agents used produced ββ, γγ, or any complex involving δ. The crosslinking experiments coupled with dissociation of virions and electron microscopic examination have shown that interactions between adjacent pentamers probably involve α and β polypeptides. The hydrophobic associations among the five αβγ structure units in a pentamer most likely involve α - α contacts. The bonding pattern within an individual structure unit is α - γ - β. Using this information, a model for the organization of individual polypeptide species in the Mengo virus capsid has been proposed.

The synthesis of Mengo virus-specific polypeptides in L-cells in the presence of the following inhibitors: tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK), zinc ions, iodoacetamide, tolylsulfonyllysyl

chloromethyl ketone (TLCK), and phenylmethylsulfonylfluoride (PMSF) has been studied. All but PMSF and TLCK were found to inhibit the post-translational cleavages of virus proteins. Carbobenzoxy-L-glutamine chloromethyl ketone (ZGCK) was synthesized and found to be a potent inhibitor of the cleavage of the Mengo capsid precursor polypeptides. This indicates a role in post-translational cleavage for a protease with specificity for glutamine residues.

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LIST OF ABBREVIATIONS

EMC virus	- encephalomyocarditis virus
ME virus	- Maus-Elberfeld virus
FMDV	- foot and mouth disease virus
HRV-1A	- human rhinovirus - 1A
RNA	- ribonucleate
mRNA	- messenger RNA
VP	- viral protein
M.W.	- molecular weight
SDS	- sodium dodecyl sulfate
MMB	- methyl-4-mercapto-butyrimidate hydrochloride
MMP	- dimethyl-3,3'-dithiobispropionimidate dihydrochloride
TNM	- tetranitromethane
TPCK	- tolylsulfonyl-phenylalanyl-chloromethyl ketone
TLCK	- tolylsulfonyl-lysyl-chloromethyl ketone
PMSF	- phenylmethylsulfonylfluoride
cpm	- counts per minute
rpm	- revolutions per minute
mA	- milliampere
µCi	- microcurie
µM	- micromolar
pfu	- plaque-forming units
IAA	- iodoacetamide
DMSO	- dimethylsulfoxide

LIST OF ABBREVIATIONS (continued)

DMA	- dimethyladipimidate
DMS	- dimethylsuberimidate
DSP	- dithiobis(succinimidyl propionate)
PBS	- phosphate buffered saline
ZGCK	- carbobenzoxy-L-glutamine chloromethyl ketone
THF	- tetrahydrofuran
PPO	- 2,5-diphenyloxazole
BME	- Eagle's basal medium

All temperatures are in degrees Celsius.

I. AN EXAMINATION OF MENGO VIRUS CAPSID STRUCTURE

Introduction

In 1963, the International Enterovirus Study Group coined the term picornavirus to describe a group of small icosahedral viruses. The picornaviruses (pico meaning small and rna signifying the type of nucleic acid in the genome) by definition are 150 to 300 Å in diameter, are non-enveloped, contain single stranded RNA genomes, and are of animal origin. The picornavirus group contains two subgroups. They are: (1) Enteroviruses; and (2) Rhinoviruses (see Table 1). Available evidence suggests that all picornaviruses have very similar structure and assembly processes.

The virus used for these studies was Mengo virus. Several physical and hydrodynamic properties of Mengo virions have been determined (Scraba et al., 1967). The hydrated diameter of the spherical particle is 30 nm, the sedimentation coefficient ($S°_{20,w}$) is 151 S, the diffusion coefficient ($D°_{20,w}$) is 1.47×10^{-7} cm^2/sec, and the partial specific volume ($\bar{V}$) is 0.70 ml/g. The molecular weight of the virion, as calculated from the Svedberg equation, is $8.3 \pm 0.7 \times 10^6$. These values can probably be considered to be representative of all picornaviruses.

The genome of the picornaviruses is a single-stranded molecule of RNA, which has approximately equal amounts of each of the four bases and contains no unusual nucleotides (Newman et al., 1973). The molecular weight of the picornaviral RNA is approximately 2.5×10^6 (Rueckert, 1976). Stretches of polyadenylic acid (poly A) that are 20 to 50

VERTEBRATE PICORNAVIRUSES

Genus Enterovirus		
A. Enteroviruses:	Polio (3 Serotypes) Coxsackie A (23) Coxsackie B (6) Echo (31) Enteroviruses of mice, swine, cattle	Sedimentation coefficient ∿155 S Buoyant density (CsCl) ∿1.34 g/ml Virions stable pH 3-10 Empty capsids produced *in vivo*
B. Cardioviruses:	EMC ME Mengo Columbia-SK MM (Serologically related)	Sedimentation coefficient ∿155 S Buoyant density ∿1.34 g/ml Virions labile 5<pH<7 in the presence of 0.1 M Cl^- or Br^- No empty capsids produced *in vivo*
Genus Rhinovirus		
A. Human Rhinovirus (>113)		Sedimentation coefficient ∿155 S Buoyant density ∿1.40 g/ml Virions labile pH <5
B. Foot-and-Mouth Disease Virus (7)		Sedimentation coefficient ∿145 S Buoyant density ∿1.43 g/ml Virions labile pH<6.5
C. Equine Rhinovirus (2)		Sedimentation coefficient ∿150 S Buoyant density ∿1.45 g/ml Virions labile pH<5

Table 1. Physical properties of picornaviruses (Rueckert, 1976).

nucleotides long have been found covalently linked to the 3' end of the RNA of several picornaviruses, i.e., poliovirus (Yogo and Wimmer, 1972; Spector and Baltimore, 1975a), Rhinovirus (Nair and Owens, 1974), EMC (Gillespie et al., 1973; Porter et al., 1974; Burness et al., 1975), and Mengo virus (Miller and Plagemann, 1972; Spector and Baltimore, 1975b; Marshall and Arlinghaus, 1976). The function of the poly A is unknown, however there is evidence that it is required for infectivity of the isolated RNA (Burness et al., 1975). Recently, a very small protein has been found to be covalently linked to the 5'-end of the virion RNA of poliovirus (Flanegan et al., 1977; Lee et al., 1976; Nomoto et al., 1977), EMC (Hruby and Roberts, 1978), and FMDV (Sangar et al., 1977). Its function is unknown, however it may play a role in synthesis of the RNA (Nomoto et al., 1977; Pettersson et al., 1977) or assembly of the virus.

The virions of all picornaviruses have been found to contain approximately sixty copies of each of four different capsid polypeptides (Rueckert, 1976). Mengo contains 58-59 molecules of α (MW = 32,500), of β (MW = 29,600), of γ (MW = 23,700), and of δ (MW = 7350) (Ziola and Scraba, 1974). The Mengo virion also contains one or two molecules of D_2, which is the precursor of α and γ, and one or two molecules of ε, which is the precursor of β and δ.

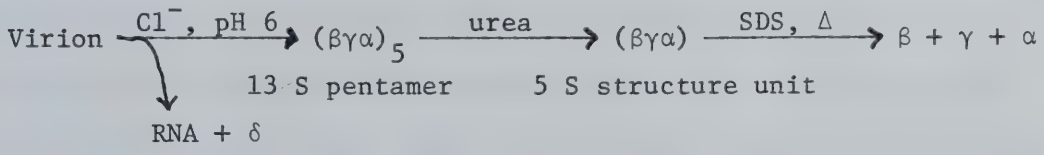
The amino acid composition data of picornavirus capsid proteins (Ziola and Scraba, 1975; Scraba et al., 1969; Rueckert and Schäfer, 1965) have shown that: (1) there are more acidic than basic residues; (2) there is a low percentage (2-3 mole %) of sulfur-containing amino acids; (3) there is a high percentage (50 mole %) of apolar residues; and (4) there is a high percentage of proline (6 to 8 mole %) and other non-α-

helix forming residues (val + ser + ile + cys + thr + gly = 40 mole %). Optical rotatory dispersion and circular dichroism studies (Scraba et al., 1967; Kay et al., 1970) indicate that there is less than 5% α-helix in the Mengo virion polypeptides in situ.

Because the capsids of the picornaviruses are compact and impermeable to the electron dense salts commonly used as negative stains in electron microscopy, surface details of the virion are not discernable in electron micrographs. Finch and Klug (1959) used X-ray crystallography to determine that the picornaviral capsid possesses icosahedral (5:3:2) symmetry, and that there must be sixty structurally equivalent asymmetric units, each with a diameter of about 65 Å. It was not until several years later that the architecture of the virus could be better determined. This was done by studying the products of controlled dissociation of ME virus (Rueckert et al., 1969; Dunker and Rueckert, 1971) Inactivation of ME occurs during incubation at pH 5.7 in the presence of 0.1 M chloride ions. This inactivation is accompanied by the release of RNA, formation of an insoluble precipitate containing all of the δ and ε polypeptides, and dissociation of the viral capsid into protein subunits with a molecular weight of 425,000 and a sedimentation coefficient of 14 S. Electrophoretic analysis showed that the 14 S particle contained equimolar amounts of the α, β, and γ polypeptides. Incubation of the 14 S particle in 2 M urea caused further dissociation into 5 S fragments having a molecular weight of 86,000 and the same polypeptide composition as that of the 14 S subunit. Rueckert et al. (1969) incorporated this data into a model of the picornaviral capsid in which the basic asymmetric structure unit is an aggregate of one molecule each of α, β, and γ (the 5 S fragment). Five

of these structural units are bonded together via hydrophobic interactions to form the 14 S pentamer. Twelve of these pentamers associate together via electrostatic bonds to form the complete capsid. The structure unit is therefore repeated 60 times in the capsid, and this corresponds to the simplest icosahedral lattice, with a triangulation number of 1 (Caspar and Klug, 1962). The requirement for twelve five-fold vertices in an icosahedral (T = 1) particle suggests that one 14 S capsomere is located at each vertex.

Similar studies with Mengo virus were also done (Mak et al., 1974), and the dissociation products were examined in the electron microscope. Upon incubation of the virion in 0.14 M chloride ions at pH 6.2, the Mengo RNA and the δ protein separated from the 13 S protein subunits, as shown in the following simplified diagram:



The 13 S subunit was found to be slightly ellipsoidal, with dimensions (length x width x thickness) of 16 x 14 x 5 nm. When the subunit was dissociated with 2 M urea, 4.7 S structure units which were about 7 nm in diameter were obtained. Both the 13 S and the 4.7 S entities contained equimolar amounts of α, β, and γ. The dimensions obtained indicate that five of the 4.7 S subunits would make up one 13 S subunit, and that twelve 13 S subunits would be required to make up one virion. The 4.7 S asymmetric structure unit conforms to the prediction of Finch and Klug. Its molecular mass is 86,000 daltons, and its diameter is 68 Å (Mak et al., 1974). It is repeated 60 times in the capsid. Of

the sixty structure units in Mengo virions, one or two presumably are "immature", i.e., have not undergone final proteolytic cleavages, and have the composition $\varepsilon\alpha\gamma$ and/or εD_2.

As yet the relationship of δ, the smallest capsid polypeptide, to the three polypeptides in the structure unit is unknown. The δ polypeptides could occupy internal positions (as suggested for bovine enterovirus by Johnston and Martin, 1971) or be clustered at the icosahedral vertices (as suggested for Coxsackievirus B3 by Philipson et al., 1973), or be grouped in trimers on the icosahedral facets to bind the $(\alpha\beta\gamma)_5$ pentamers together (as suggested for Mengovirus by Mak et al., 1974).

Other experiments have been done to further probe the structure of the picornaviral capsid. The relative location of the Mengo capsid polypeptides with respect to the external surface of the virion was investigated by measuring the relative susceptibilities of the polypeptides to lactoperoxidase-catalyzed iodination of tyrosine groups (Lund et al., 1977). When intact virions were subjected to iodination for one minute, radioactive iodine was incorporated predominantly into the tyrosines of the α polypeptides, with some incorporation into the β polypeptides. As longer incubation times were allowed, the viral capsid loosened and then disrupted; this process was accompanied by incorporation of radioactive iodine into the tyrosines of the γ and δ polypeptides. After normalizing the data for the number of tyrosine residues in each polypeptide (α - 11, β - 10, γ - 11, and δ - 3), and assuming that there is a random distribution of tyrosine residues in each of the individual polypeptides, it was concluded that about 65% of the external surface of the virion is occupied by α polypeptides, while the remaining 35% of

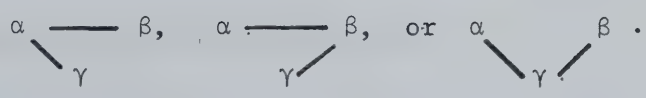
the surface is β polypeptides. The γ and δ polypeptides appeared to be buried in the interior of the virion. Studies done with FMDV (Talbot et al., 1973), bovine enterovirus (Carthew and Martin, 1974) and poliovirus (Lonberg-Holm and Butterworth, 1976) gave analogous results.

Immunodiffusion and complement fixation experiments using antisera prepared against the individual capsid polypeptides (Lund et al., 1977) confirmed the iodination results. In both tests, only serum containing antibodies for the α or β polypeptides was capable of complexing with intact virions.

Lund et al. (1977) further showed, by use of plaque neutralization and hemagglutination-inhibition tests, that the α polypeptides are responsible for attachment of Mengo virions to cellular receptors. In the case of FMDV, the analogous VP1 polypeptides perform this function (Rowlands et al., 1971). Thus, even though both the α and β polypeptides are on the external surface of the virion, only the α polypeptides are responsible for virus-cell interaction.

The first chapter of this thesis is concerned with experiments using chemical cross-linkers to examine the relative positions of the Mengo capsid polypeptides with respect to one another. The existence of three non-identical polypeptide chains in a subunit could imply either that the three polypeptides are intermeshed about each other to form a single trimeric unit, or that each polypeptide has a specific and discrete location, related by specific bonding patterns to its nearest neighbors. If α, β and γ each occupy their own discrete domain within the structure unit, then crosslinking would be expected to link together specific dimers or trimers of α, β, and γ, depending on which polypeptides were close enough to each other to have noncovalent bonding

contacts. The noncovalent bonding pattern within the 5 S structure unit could be:



On the other hand, if α, β, and γ are intermeshed in a single trimeric unit, then crosslinking would be expected to link together all 3 peptides into  and $(\alpha,\beta,\gamma)_n$ complexes.

It was also hoped that the location of the hydrophobic bonding contacts between structure units and the ionic bonding contacts between pentamers could be determined. Since the structure unit contains only one molecule each of α, β, and γ, the formation of such crosslinked complexes as α_n, β_n, or γ_n would be indicative of specific contacts between structure units in the 13 S pentamer. With respect to the δ polypeptides, could these be demonstrated to exist either in trimers as suggested by Mak et al. (1974), or to be clustered in pentamers as proposed by Philipson et al. (1973)?

Materials and Methods

Virus. The M-Mengo plaque variant, originally isolated by Ellem and Colter (1961) was used throughout these studies. The propagation of virus in L-cells, radioactive labeling, and purification of the virus was carried out according to the procedures described by Ziola and Scraba (1974).

Crosslinking reagents. Dimethylsuberimidate dihydrochloride (DMS) was obtained from Aldrich Chemical Company, Inc. Dimethyl adipimidate dihydrochloride (DMA) and dithiobis(succinimidyl propionate) (DSP) were obtained from Pierce Chemical Co.

Crosslinking of the Mengo virion with DMS or DMA. Proteins in intact Mengo virions were crosslinked using DMS or DMA at varying concentrations in 0.2 M triethanolamine buffer, pH 8.5, for 24 hours at room temperature. The crosslinked virions were then made 2% in SDS and 5% in mercaptoethanol and boiled for five minutes to solubilize the polypeptides. More complete details are given in the figure legends.

Crosslinking of the Mengo virion with DSP. Mengo virions were suspended in 0.2 M triethanolamine buffer at pH 8.5. DSP was dissolved in dimethylsulfoxide (100 mg/ml) before addition to the virions. Incubation was allowed for 24 - 48 hours at room temperature. The virions were then made 2% in SDS and boiled for five minutes to disrupt the capsid. More complete details are given in the figure legends.

SDS polyacrylamide gel electrophoresis of capsid polypeptides. Tube gels consisted of 7.5% acrylamide, 0.2% N,N'-methylenebisacrylamide and 0.1% SDS, in 0.1 M sodium phosphate, pH 7.2. Polymerization was catalyzed by N,N,N',N'-tetramethyl ethylenediamine and ammonium persulfate, at final concentrations of 0.083% and 0.042%, respectively. The crosslinked viral polypeptides were prepared as above, and to 100 µl samples containing 100 µg of viral protein were added 10 µl of 50% glycerol and 5 µl of 0.05% bromophenol blue in electrophoresis buffer (0.1 M sodium phosphate, pH 7.2, containing 0.1% SDS). The samples

were layered onto 20 x 0.5 cm gels and electrophoresed for 15 - 18 hours at 5 - 7 mA per gel. After electrophoresis, the gels were stained in Coomassie blue according to the method of Weber and Osborn (1969).

Two dimensional SDS polyacrylamide gel electrophoresis of crosslinked complexes. Slab gels of the same composition as the tube gels were prepared so that they were 0.75 mm thick, and had wells 8 mm wide. Virions which had been crosslinked as above were electrophoresed at 25 mA until the bromophenol blue which was used as a marker had run down five inches. The appropriate strip was then cut from the slab gel and soaked in a cleavage solution of 10% mercaptoethanol in electrophoresis buffer for 30 minutes for DSP-crosslinked virions, or a solution of 15 parts ammonium hydroxide, one part acetic acid, 1% SDS, and 1% mercaptoethanol (Bickle et al., 1972; Barritault et al., 1975) for 24 hours for DMS crosslinked virions. After cleavage, the strips were soaked in electrophoresis buffer for 30 minutes, followed by soaking in a solution of 10% acrylamide, 0.2% N,N'-methylenebisacrylamide, 0.1% SDS, 0.1 M sodium phosphate (pH 7.2) and 0.083% N,N,N',N'-tetramethylethylenediamine for 10 minutes. The first dimension strips were laid horizontally across the top of a 1.5 mm thick 10% slab gel, and acrylamide solution was used to polymerize the first dimension gel into place. Electrophoresis was performed for 18 hours at a current of 50 mA, after which the slab gels were prepared for autoradiography using the method of Bonner and Laskey (1974). This procedure involved the following steps: (1) dehydration of the slab gels by soaking twice in dimethylsulfoxide for 30 minutes, (2) soaking for 3 hours in 22.2% (w/v) PPO in DMSO, (3) rehydration for 45 minutes in 3% glycerol, (4) drying the gel with heat

under vacuum, and (5) exposing the dried gel to Kadak X-omat X-ray film in the dark at -70° for the required time (4 days to 3 weeks).

Results

Chemical crosslinkers have been used to study spatial relationships among proteins in a number of different complex assemblies (Peters and Richards, 1977). In an attempt to crosslink the peptides in the Mengo virion capsid, several reagents were tried, the most successful being dimethylsuberimidate, DMS (Davies and Stark, 1970; Garoff, 1974), dimethyladipimidate, DMA (Hartman and Wold, 1967; Expert-Bezançon et al., 1977), and dithiobis (succinimidyl propionate), DSP (Bragg and Hou, (1975). All three of these compounds react with free amino groups in polypeptides, i.e., the N-terminal residue of the polypeptide or the ε-amino group of lysine residues within the polypeptide (see Figure 1). The α polypeptide of the Mengo capsid has 15 lysine residues, β has 7, γ has 10, while δ has only 2 lysines and a blocked N-terminal residue (Ziola and Scraba, 1976). Thus, it was expected that δ might be difficult to crosslink, and this was indeed found to be the case.

When Mengo virions were crosslinked with 7 mM DMS or DMA, high molecular weight complexes were obtained (see Figure 2). Graphical analysis of the polyacrylamide gels (Figure 3) indicated that the crosslinked complexes formed had molecular weights of 54,000, 58,000, 65,000, 86,000, and 95,000. Because of the similarity of structure of DMS and DMA it was assumed that the two crosslinked complexes formed with DMA were identical to the 58,000 and 65,000 complexes formed with DMS. By increasing the concentration of DMS to 25 mM it was possible to see two

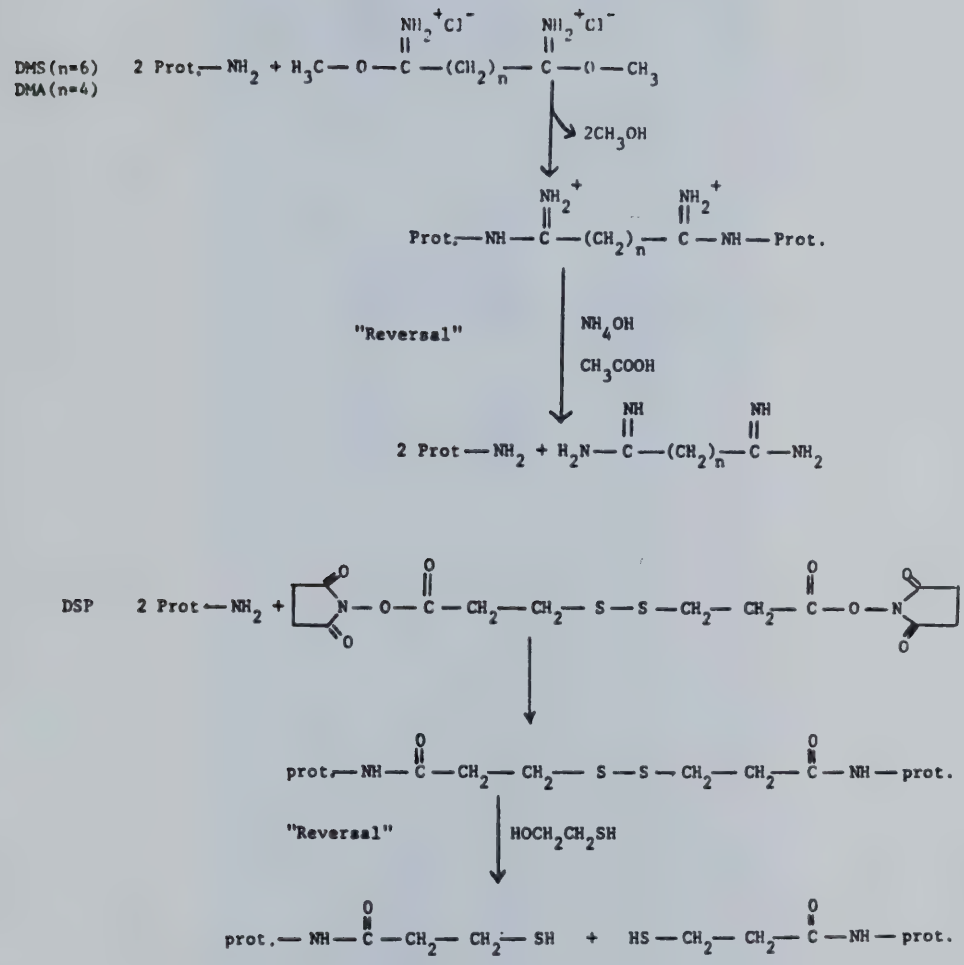


Figure 1. The reactions of dimethylsuberimidate (DMS), dimethyl-adipimidate (DMA), and dithiobis(succinimidyl propionate) (DSP).

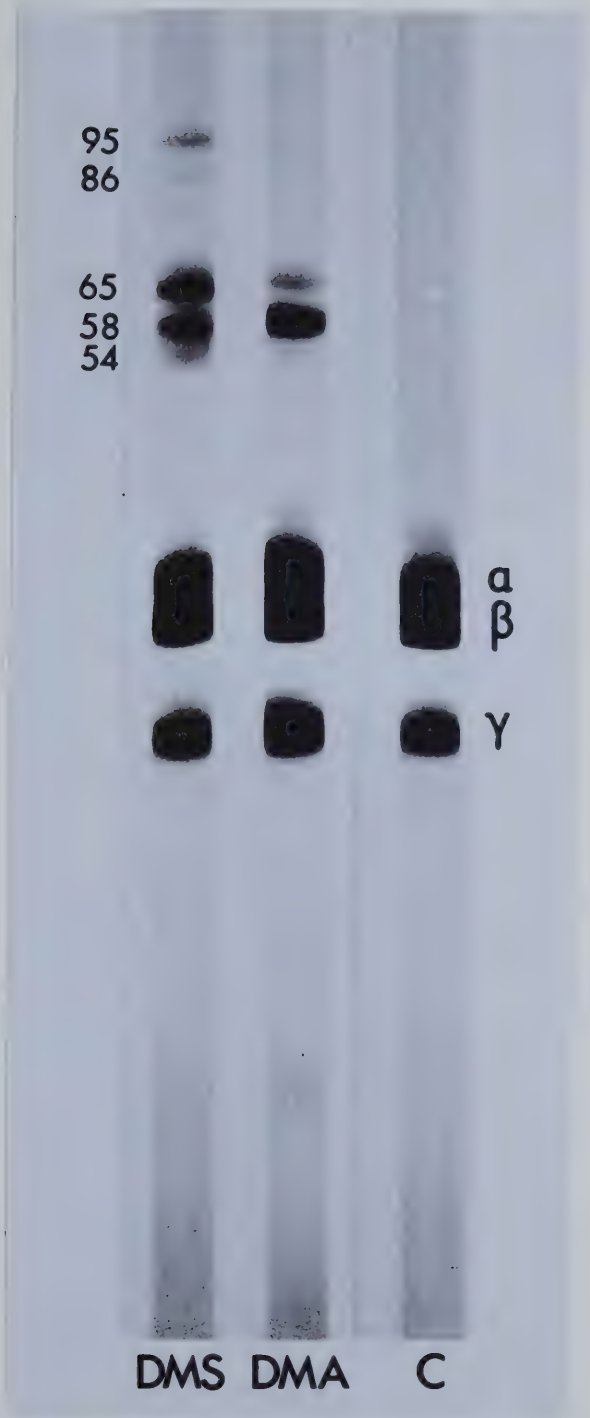


Figure 2. Crosslinking Mengo virions with DMS and DMA. Mengo virions (1 mg protein/ml) were crosslinked with 7 mM DMS, or 7 mM DMA in 0.2 M triethanolamine buffer, pH 8.5. The incubation continued for 24 hours at room temperature. The virions were then disrupted, electrophoresed, and the gels were stained according to the procedure of Weber and Osborn (1969). The electrophoretic profile of the polypeptides from untreated virus is shown in C. The δ polypeptides stain very poorly with Coomassie Blue because they each have only two basic amino acid (lysine) residues. The molecular weights (in thousands) of the crosslinked complexes were determined by graphical analysis (see Figure 3) and are indicated.

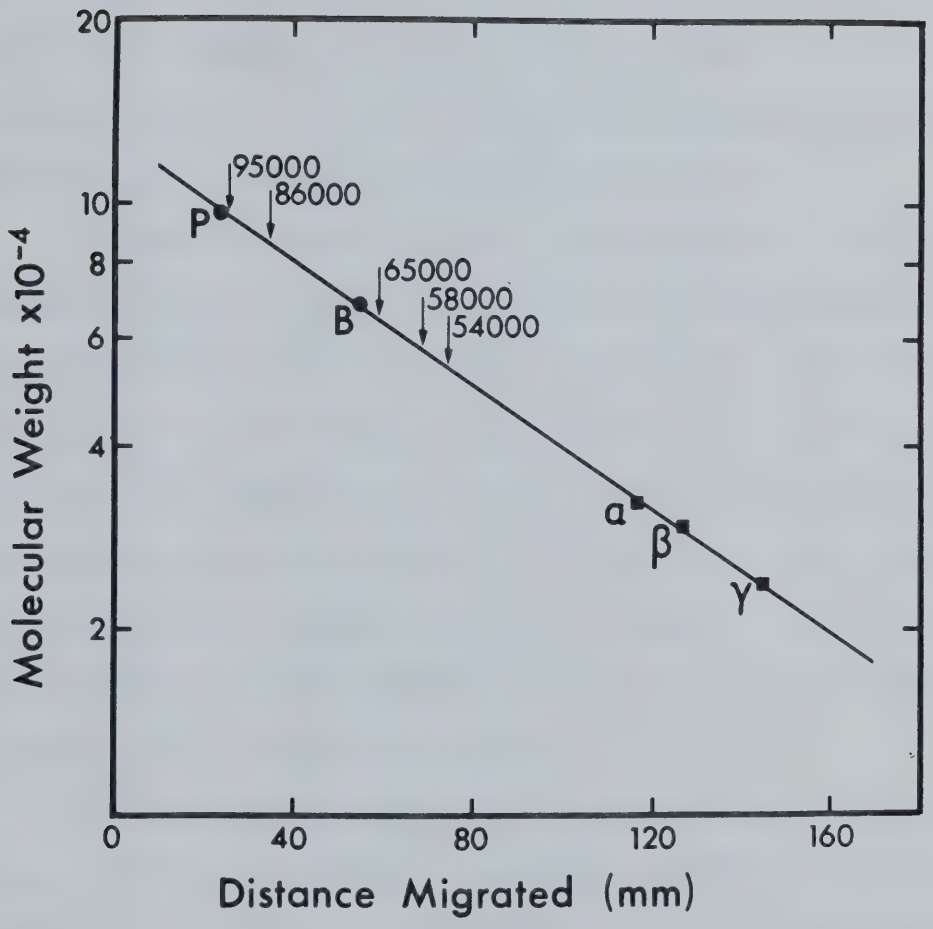


Figure 3. Molecular weight determination by SDS-gel electrophoresis of the crosslinked complexes obtained with DMS and DMA. The capsid polypeptides α (MW = 32,500), β (MW = 29,500), and γ (MW = 23,700) were used as internal standards. Bovine serum albumin (B; MW = 68,000) and phosphorylase b (P; MW = 95,000) marker proteins were electrophoresed concurrently in a separate gel.

more complexes with higher molecular weights (see Figure 4). Graphical analysis indicated that their molecular weights were about 115,000 and 135,000; however, these values are at the limits of accuracy possible with 7.5% polyacrylamide gels (Dunker and Rueckert, 1969).

If the virions were disrupted by boiling in SDS and mercaptoethanol prior to incubation with DMS or DMA, no crosslinked complexes were formed. Therefore, the crosslinked complexes that are formed in the Mengo capsid are probably a reflection of capsid structure, and not simply a result of random collisions between virions.

In order to obtain a positive identification of the crosslinked complexes obtained with DMS, a two-dimensional electrophoresis system was employed (described in Materials and Methods). Figure 5 shows the results of such an experiment. From this gel, it was positively determined that the 58,000 molecular weight band was $\alpha\gamma$, the 65,000 molecular weight band was α_2, and the 95,000 molecular weight band was α_3. The 54,000 molecular weight band is most likely $\beta\gamma$, and the 86,000 band is most likely $\alpha\beta\gamma$, but these were not present in large enough amounts to produce spots in the autoradiogram.

Similar experiments have been done using DSP as the crosslinking reagent. As shown in Figure 6, incubation of Mengo virions with DSP produced complexes with molecular weights equal to 52,000, 62,000, 86,000, 95,000, and $\sim$120,000 (the latter being at the limits of accuracy of a 7.5% gel). The DSP complexes were subsequently subjected to cleavage and two-dimensional SDS-gel electrophoresis. From the slab gel shown in Figure 7, the 52,000 molecular weight complex was identified as $\beta\gamma$, the 62,000 molecular weight complex as $\alpha\beta$, and the 86,000 molecular weight complex as $\alpha\beta\gamma$. In another experiment (see Figure 8) the

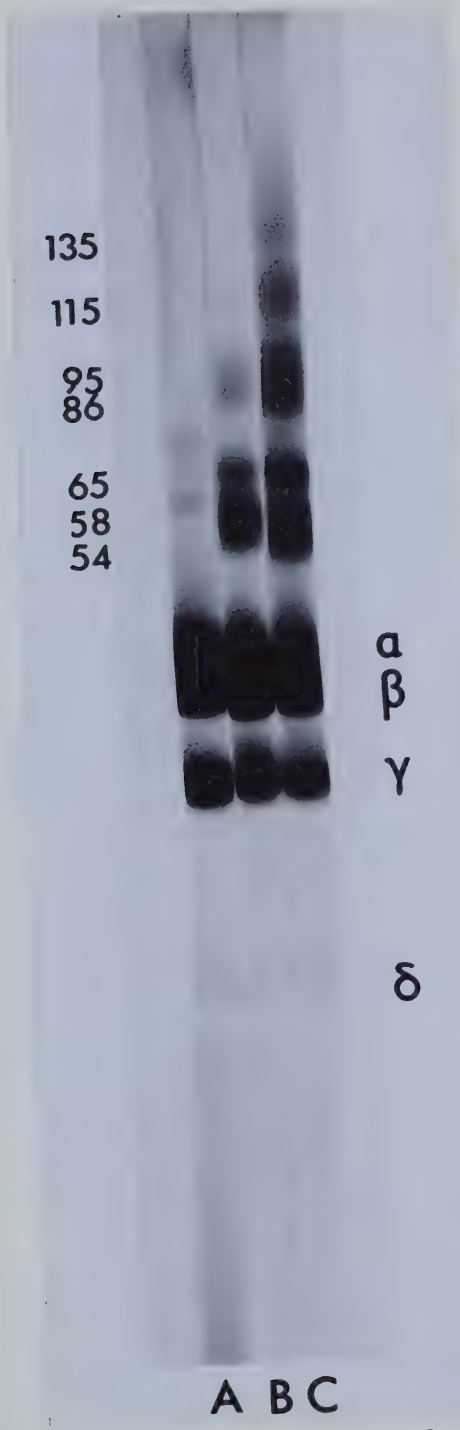


Figure 4. Gel electrophoretic demonstration of crosslinked complexes obtained with 25 mM DMS. Virions (1 mg protein/ml) were incubated with 25 mM DMA (B) or 25 mM DMS (C), in 0.2 M triethanolamine, pH 8.5, for 24 hours prior to solubilization and electrophoresis. The profile of untreated virions is shown in A. The molecular weights (in thousands) of the polypeptides are indicated.

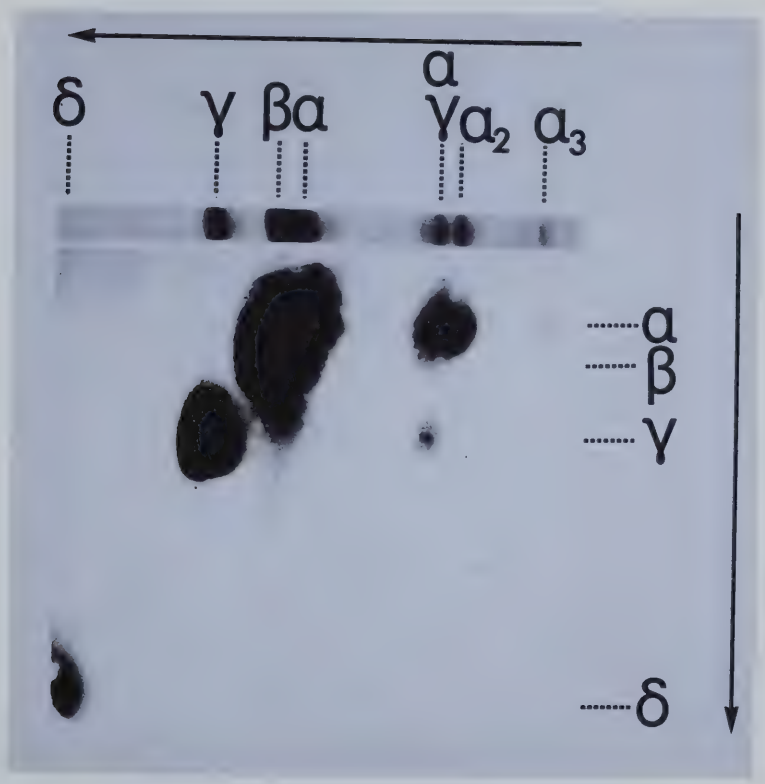


Figure 5. Cleavage and identification of DMS crosslinked complexes. The composition of the complexes obtained after crosslinking with DMS was determined using the two dimensional SDS polyacrylamide gel system described in Materials and Methods. Virions (2 mg protein/ml) were incubated with 7 mM DMS for 24 hours, disrupted, and subjected to SDS-polyacrylamide gel electrophoresis in the first dimension. A photograph of one such gel, with protein bands made visible by Coomassie Blue staining, is shown at the top of the Figure. An identical gel was treated with NH_4OH and CH_3COOH to cleave the crosslinks, then laid horizontally across the top of the second dimension slab gel such that the higher molecular weights complexes were to the right. The direction of electrophoresis in the second dimension is indicated by the arrow. After drying the gel, ^3H-labelled protein spots were visualized by autoradiography.

Figure 6. Gel electrophoretic demonstration of crosslinked complexes obtained with DSP. Virions (2 mg protein/ml) were incubated with 7 mM DSP (A) or 0.7 mM DSP (B) in 0.2 M triethanolamine (pH 8.5) for 48 hours. The samples were solubilized, electrophoresed on a 0.75 mm wide 7.5% slab gel, and the gel was stained with Coomassie Blue. The profile of untreated virions is shown in C. The molecular weights (in thousands) of the polypeptides are indicated.

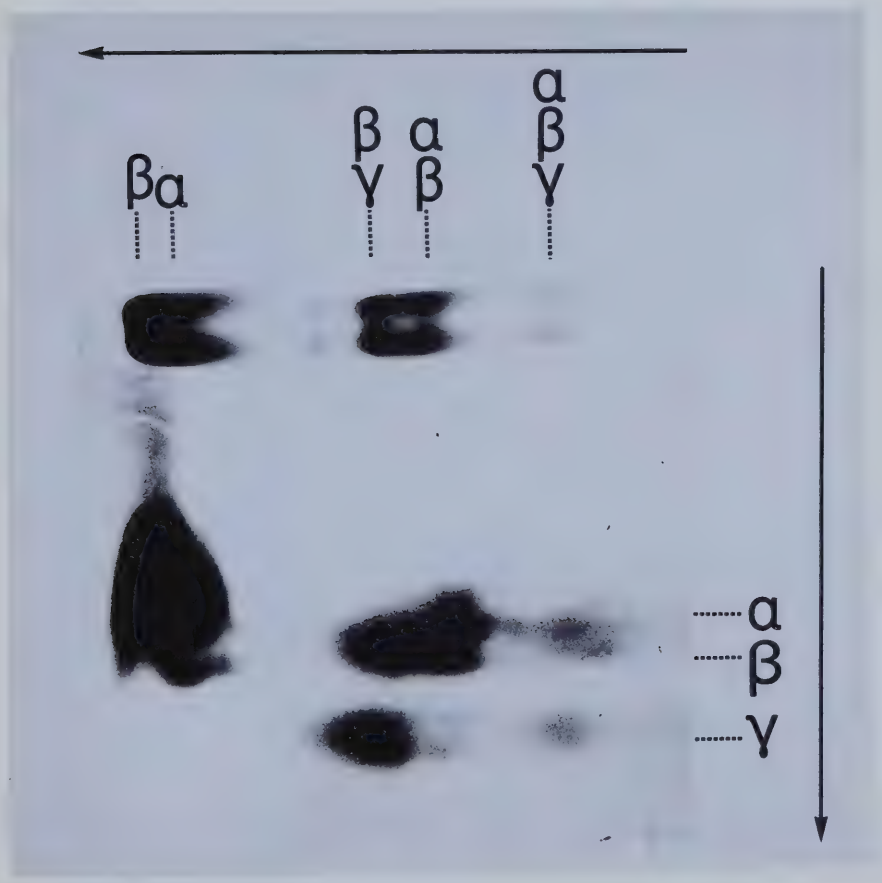


Figure 7. Identification of DSP crosslinked complexes. Virions (3 mg protein/ml) were incubated with 7 mM DSP for 24 hours at room temperature. They were then made 2% with SDS and boiled for 5 minutes to disrupt the capsid. The procedure for two-dimensional electrophoresis was as described in Materials and Methods.

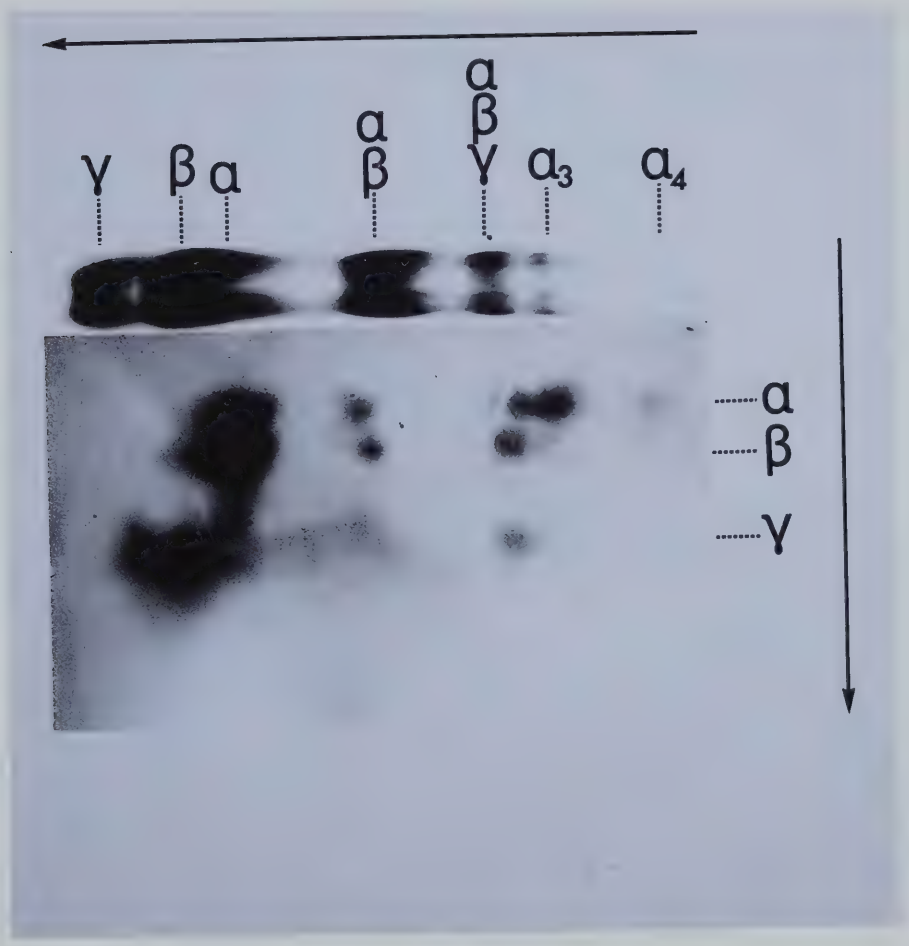


Figure 8. Identification of DSP crosslinked complexes. Virions (2 mg protein/ml) were incubated with 13 mM DSP for 48 hours at room temperature. The samples were then disrupted and applied to the two-dimensional electrophoretic system described in Materials and Methods.

concentration of DSP was increased and the 97,000 molecular weight complex was identified as α_3. The ∿120,000 molecular weight complex was identified as α_4; therefore its actual molecular weight is 4 x 32,500 = 130,000. At this higher concentration of DSP, the βγ complex was incorporated into an αβγ complex, whereas the αβ complex was unaffected. This indicates that β - γ linkages occurred within an αβγ structure unit.

A summary of all the crosslinked complexes obtained with DMS, DMA, and DSP is given in Table 2.

In order to determine which, if any, of the crosslinks formed with DMS, DMA, and DSP were between 13 S pentamers, crosslinked virions were subjected to dissociation by incubation with 0.14 M NaCl in 0.02 M sodium phosphate (pH 6.2) and examined in the electron microscope. Figure 9 shows that virions that had been crosslinked with DMS or DMA were easily dissociated into 13 S pentamers. However, as shown in Figure 9e, virions which had been crosslinked with DSP were resistant to dissociation. These capsids are obviously under a disruptive influence, but the crosslinks induced by DSP are capable of holding several of the pentamers together.

Attempts to crosslink the Mengo virion with other reagents were made. One of these attempts involved the use of tetranitromethane (TNM) to nitrate tyrosine residues in the capsid polypeptides (Sokolovsky et al., 1966) followed by reduction of the new nitro groups with sodium hydrosulfite ($Na_2S_2O_4$) (Sokolovsky et al., 1967). This procedure was followed by incubation with DMS for 24 hours at room temperature, which was intended to form crosslinks involving the new amino groups in the peptides. However, no different crosslinked complexes were detected. An attempt was made to crosslink Mengo capsids using polylysine (Gordon et al., 1974),

CHEMICAL CROSSLINKING OF POLYPEPTIDES IN THE MENGO VIRION

Crosslinking Reagent	Maximum Linkage Distance (nm)	Molecular Weight of Complex[a]	Probable Composition	Composition Confirmed[b]
Dimethyladipimate (DMA)	0.8	58,000	αγ	
		65,000	α_2	
Dimethylsuberimidate (DMS)	1.1	54,000	βγ	
		58,000	αγ	yes
		65,000	α_2	yes
		86,000	αβγ	
		95,000	α_3	yes
Dithiobis(succinimidyl propionate) (DSP)	1.5	54,000	βγ	yes
		62,000	αβ	yes
		86,000	αβγ	yes
		97,000	α_3	yes

[a] Molecular weights estimated from migration during electrophoresis in SDS-polyacrylamide gels.

[b] Composition confirmed by chemical "reversal" of the crosslinks followed by electrophoresis in the second dimension.

Table 2. Crosslinked complexes obtained with DMS, DMA, and DSP.

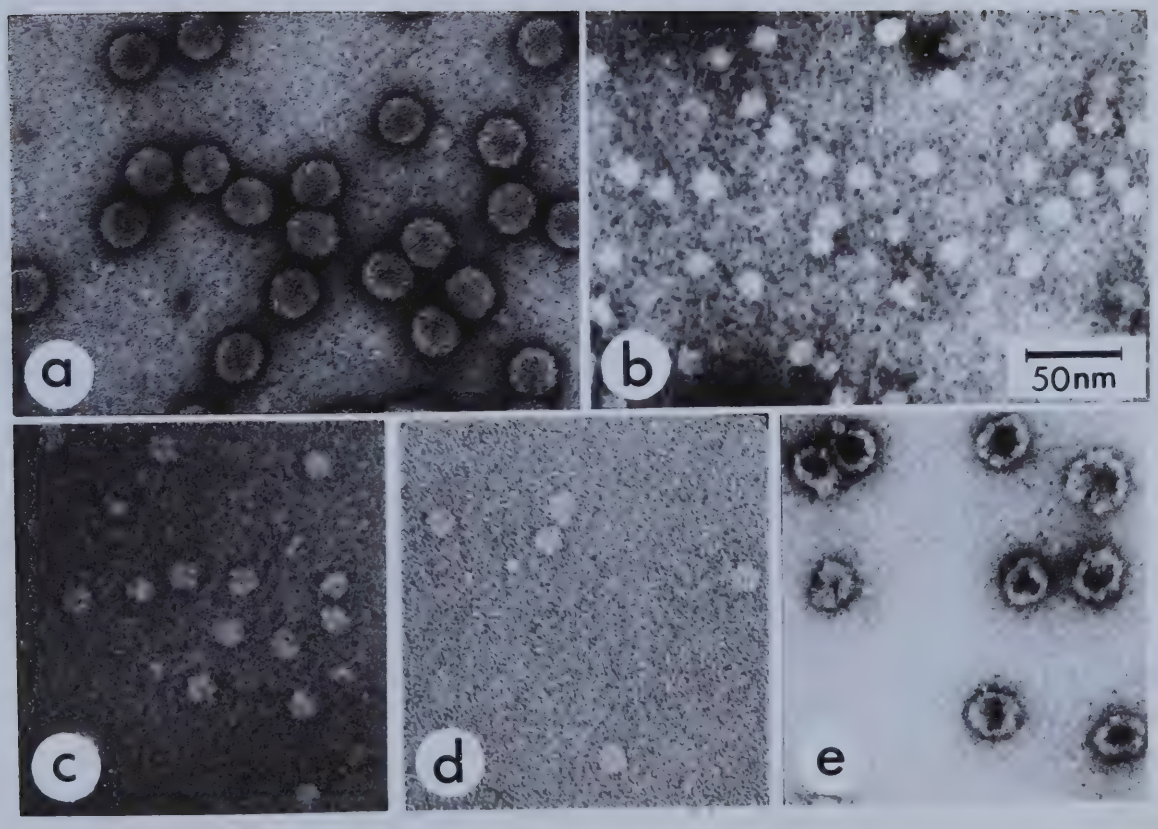


Figure 9. Controlled dissociation of crosslinked virions. Whole virions (a) were dissociated into 13 S pentamers (b) by treatment with 0.14 M NaCl, in 0.02 M sodium phosphate, pH 6.2, at 56° for 30 minutes. Crosslinked virions (2 mg protein/ml) were incubated with 7 mM DMA (c), 25 mM DMS (d), or 7 mM DSP (e) prior to dissociation. All samples were negatively stained with 1% uranyl formate and photographed in the electron microscope.

but no crosslinked complexes could be observed. Crosslinking experiments with dimethyl 3,3'-dithiobis propionimidate (MMP; (Wang and Richards, 1974) and methyl-4-mercaptobutyrimidate (MMB; Traut et al., 1973; Sommer and Traut, 1975), both specific for amino groups in proteins, were tried. MMP was found to be unreactive. MMB initially produced crosslinked complexes with molecular weights of 58,000 ($\alpha\gamma$?), 64,000 (α_2?) and 90,000 (α_3?); however, several attempts to duplicate this experiment were unsuccessful.

Discussion

Any model that is made of the capsid structure of the Mengo virion must be consistent with all of the experimental data that have been obtained thus far. Lund et al. (1977) have published data pertaining to the distribution of the Mengo capsid polypeptides with respect to the surface of the virus particle. Their results indicated that: (1) the α polypeptide occupies about 65% of the external surface of the virion, (2) the γ and δ polypeptides are within the interior of the virion, (3) the β polypeptide is partially exposed on the exterior of the virion, and (4) the α polypeptide is responsible for attachment to cells.

Dissociation studies (Dunker and Rueckert, 1971; Mak et al., 1974) have shown that the Mengo capsid must have twelve pentamers located at the twelve vertices of an icosahedron. The pentamers are held together by electrostatic interactions and dissociated from each other in the presence of chloride ions and low pH. Each pentamer is made up of five identical structure units, which are held together by hydrophobic interactions. The crosslinking data now gives the identity of the polypeptides involved in each of these noncovalent bonding interactions.

Since the structure unit contains one molecule each of α, β, and γ, the α_2, α_3, and α_4 crosslinked complexes must have been formed between different structure units. The five structure units in the capsomere must be positioned such that their α polypeptides are in contact with one another. Therefore, the only possible positioning for structure units is such that the five α polypeptides are together at the vertices of the capsid.

DMS and DMA crosslinked virions could dissociate into pentamers when incubated with chloride ions at low pH. However, DSP crosslinked virions could not be dissociated. αβ is the only crosslinked complex made by DSP and not made by DMS or DMA (refer to Table 2). Therefore, it is postulated that in the Mengo capsid, electrostatic interactions between the α and β polypeptides hold adjacent pentamers together; these interactions are disrupted by chloride ions at pH 6.2.

DMS and DSP also produce α - γ and β - γ crosslinks. The existence of these complexes indicates that the bonding pattern within the structure unit is α - γ - β. Each of the three polypeptides within a structure unit occupies its own discrete domain, as opposed to them being intermeshed about each other.

Figure 10 shows a model of the Mengo capsid which is consistent with various experimental data obtained thusfar:

1) Five αβγ structure units cluster together at each of the twelve vertices of an icosahedron to form the 13 S pentamer (Dunker and Rueckert, 1971; Mak et al., 1974).

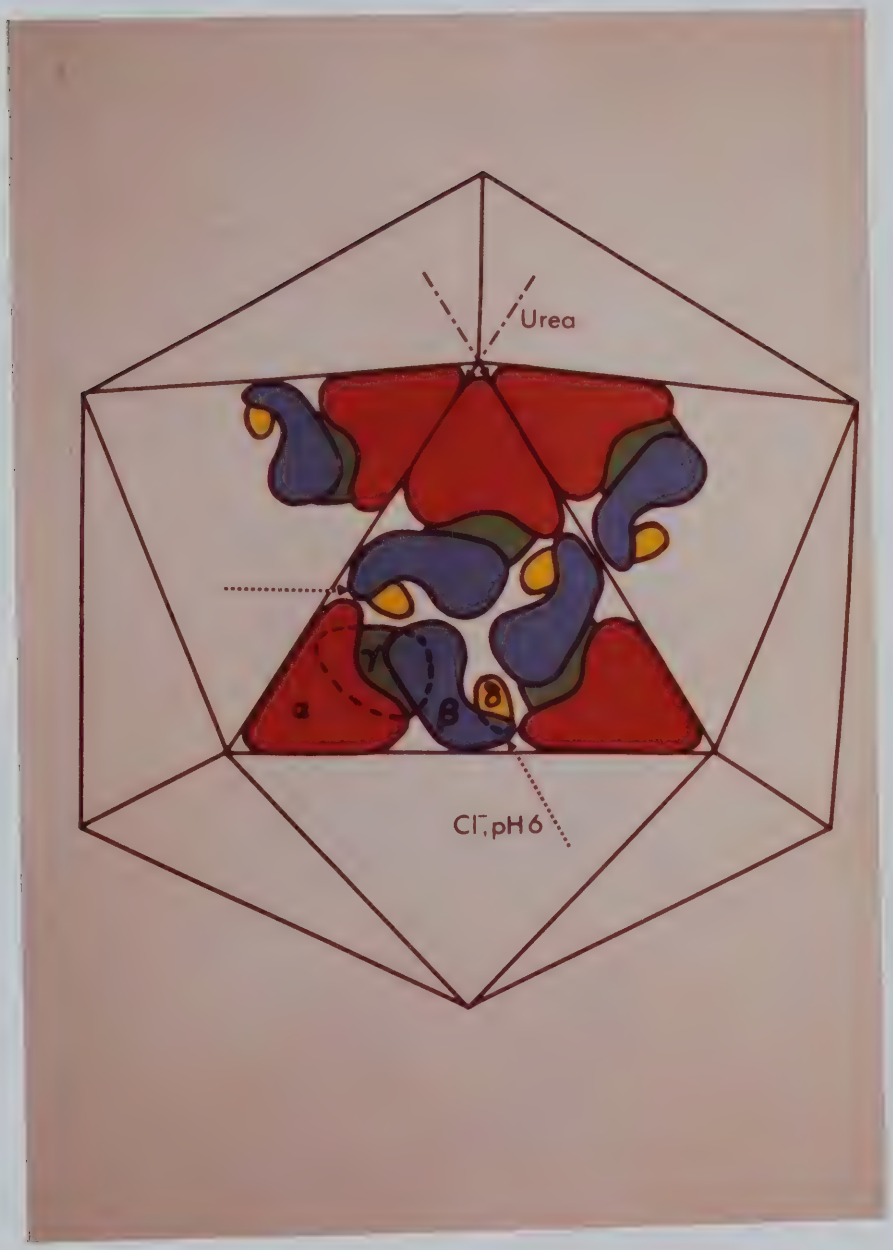


Figure 10. A model of the Mengo virus capsid. The shapes of individual polypeptides are arbitrary. This is a surface view; the thickness of the capsid is 5 - 7 nm (Mak et al., 1974). Arrows indicate the non-covalent interactions disrupted by chloride ions (pH 6) and by urea.

2) The α polypeptides, which are responsible for attachment of the virion to a susceptible cell, occupy ∿65% of the exterior surface of the virion, with parts of the β polypeptide also being exposed. The γ and δ polypeptides are buried within the capsid (Lund et al., 1977).

3) Crosslinking experiments (discussed above) have shown that the electrostatic interactions between adjacent pentamers probably involve contacts between α and β polypeptides. The hydrophobic interactions between the five αβγ structure units in a pentamer most likely involve α - α contacts. The bonding pattern within an individual structure unit is α - γ - β.

4) The gene order of the Mengo virus capsid polypeptides is $^{N}\delta - \beta - \gamma - \alpha^{C}$ (Paucha et al., 1974).

The model is still incomplete in that the location and structural role of the δ polypeptides remains mysterious. All that is known about δ is that it is in the interior of the capsid. However, it has been demonstrated that during virion dissociation by chloride ions at pH 6, the δ polypeptides are precipitated together with the viral RNA. Since δ has an internal location in the capsid (Lund et al., 1977) and since it is derived by the cleavage of ε → β + δ as the RNA is incorporated into a new virion, it is not inconceivable that δ is located as shown in Figure 10 and is in intimate contact with the RNA.

II. CLEAVAGE OF MENGO VIRUS PROTEINS *in vivo*

Introduction

Studies of the translation of picornaviral proteins *in vivo* indicate that the virion RNA - after the removal of Vg - functions as mRNA (Penman *et al.*, 1964; Summers and Levintow, 1965; Nomoto *et al.*, 1977). Soon after the initiation of viral infection there is a decrease in host cell protein synthesis such that by three to four hours after infection it is virtually stopped. This phenomenon has permitted the specific labeling of viral-coded proteins in infected cells. The RNA of poliovirus has a molecular mass of approximately 2.6×10^6 daltons, and is therefore able to code for about 270,000 daltons of protein. Analysis of the viral proteins synthesized by poliovirus (Summers *et al.*, 1965) indicated that both capsid and noncapsid viral proteins were synthesized; however the sum of all their molecular weights was far greater than that expected. This fact was explained by use of pulse-chase experiments in which the infected cells were exposed to radioactive amino acids for a brief period of time, then the labeled amino acids were removed from the medium and the cells were allowed to continue synthesizing viral polypeptides. This experiment demonstrated a flow of radioactivity from the larger polioviral polypeptides synthesized during the pulse to the smaller peptides present at later times (Summers and Maizel, 1968; Maizel and Summers, 1968; Jacobson and Baltimore, 1968; Jacobson *et al.*, 1970; Summers *et al.*, 1971). Similar experiments done with Coxsackie virus (Holland and Kiehn, 1968),

cardioviruses (Butterworth et al., 1971; Dobos and Martin, 1972; Paucha et al., 1974; Lucas-Lenard, 1974; Ginevskaya et al., 1972), rhinoviruses (McLean and Rueckert, 1973), and FMDV (Van de Woude et al., 1972; LaPorte and Lenoir, 1972; Black, 1975) indicated that cleavage of larger viral precursor proteins into smaller peptides occurred with all the picornaviruses.

The picornaviral mRNA has a single initiation site (Oberg and Shatkin, 1972). Jacobson and Baltimore (1968) suggested that the entire genome of poliovirus is translated as a single unit to yield a large precursor polypeptide ("polyprotein") from which all viral proteins are produced by subsequent cleavages. Normally the polyprotein is unstable in vivo; however it was shown that if the polyprotein was synthesized in the presence of amino acid analogs such as p-fluorophenylalanine, canavanine (arginine analog), azetidine-2-carboxylic acid (proline analog), and ethionine (methionine analog), it was no longer susceptible to cleavage, and could be observed in polyacrylamide gels. The effect of amino acid analogs was tested in uninfected cells, and it was found that they had no effect on the size of host proteins (Jacobson and Baltimore, 1968). Therefore, the initiation and termination of protein synthesis occurs properly in the presence of the analogs. These must be incorporated into the viral polyprotein in infected cells and alter its configuration so that it is no longer susceptible to proteolysis. Experiments done with other picornaviruses also found that accumulation of polyprotein by inhibition of proteolytic cleavages could be obtained by use of amino acid analogues (Paucha et al., 1974; Abraham and Cooper, 1975a; Dobos and Martin, 1972; Black, 1975; Collins

and Roberts, 1972), protease inhibitors (Korant, 1972; Summers et al., 1972; Jacobson et al., 1970; Lucas-Lenard, 1974; Black, 1975), zinc ions (Butterworth and Korant, 1974; Korant and Butterworth, 1976; Nakai and Lucas-Lenard, 1976; Korant et al., 1974), elevated temperatures (Dobos and Martin, 1972), or iodoacetamide (Korant, 1973).

Based on information obtained from the kinetics of synthesis and cleavage (Jacobson and Baltimore, 1968; Summers and Maizel, 1968) pactamycin mapping (Butterworth, 1973; Taber et al., 1971; Summers and Maizel, 1971; Oberg and Shatkin, 1972), and tryptic peptide analysis (Summers and Maizel, 1968; Jacobson and Baltimore, 1968; Jacobson et al., 1970; Abraham and Cooper, 1975b) the cleavage scheme for poliovirus proteins was determined. Studies of the cleavage patterns of cardioviruses (Ginevskaya et al., 1972; Dobos and Martin, 1972; Dobos and Plourde, 1973; Esteban and Kerr, 1974; Lucas-Lenard, 1974; Paucha et al., 1974), rhinoviruses (McLean and Rueckert, 1973), FMDV (Van de Woude and Ascione, 1974; Black, 1975) have shown remarkable similarity. The cleavage scheme for Mengo viral proteins determined by Paucha et al. (1974) is given in Figure 11 and can be considered to be representative of the picornaviruses.

Available evidence indicates that the polyprotein is cleaved as it is being synthesized. Jacobson et al. (1970) measured the size distribution of polysome-bound nascent polypeptides of poliovirus-infected cells and could not detect polypeptides of higher molecular weight than 130,000. Experiments done with EMC virus (Butterworth and Rueckert, 1972) showed that the precursor of the capsid proteins, A,

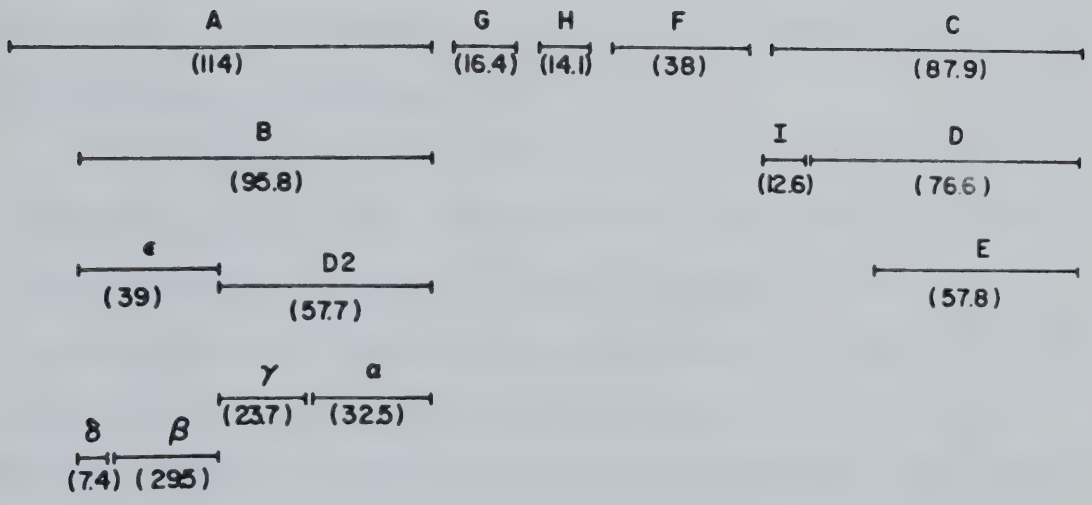


Figure 11. Proposed scheme for the cleavage of Mengo virus-specific polypeptides. The numbers in brackets refer to the molecular weight, in thousands, of each polypeptide.

could be labeled and released in about 2 minutes. Since it takes up to 10 minutes for the ribosome to traverse the entire length of the viral RNA molecule (Rekosh, 1972), the capsid precursor must be released independently soon after its synthesis. As shown in Figure 11, primary cleavages of the Mengo polyprotein which occur during its synthesis produce three stable noncapsid polypeptides (G, H, F), the capsid precursor polypeptide (A) and a viral RNA replication complex precursor (C). Secondary cleavages of these proteins probably occur on cytoplasmic membranes and may be catalyzed by a virus-specified protease (Korant, 1972; Korant, 1973). The morphogenetic cleavage of $\varepsilon \rightarrow \beta + \delta$ accompanies the encapsidation of the viral RNA (Jacobson and Baltimore, 1968; McGregor and Rueckert, 1977).

Inhibition of the proteolytic steps involved has been used to examine the details of the post-translational processing of picornaviral polypeptides. Korant (1972) used tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK) and tolylsulfonyllysyl chloromethyl ketone (TLCK), inhibitors of chymotrypsin and trypsin respectively (Shaw, 1967) to inhibit the primary cleavages of polioviruses. The effectiveness of the inhibitor varied depending on the cell line chosen; polioviral precursor proteins with molecular weights larger than those of the products of primary cleavage were accumulated in infected monkey kidney cells treated with TPCK and in HeLa cells treated with TLCK. This suggested that the viral polyprotein is cleaved, at least in part, by a cellular enzyme or enzymes. The viral precursor proteins obtained by use of the inhibitors were labeled and extracted, and could be partially cleaved <u>in vitro</u> by either trypsin or chymotrypsin into lower molecular weight proteins resembling viral polypeptides. If

the polyprotein was denatured before digestion with these proteases, extensive cleavage occurred, and a large number of low molecular weight polypeptides was formed. This indicated that the native polyprotein must have a configuration that exposes only certain sites for primary cleavage by host enzymes. Because both TLCK and TPCK react irreversibly with proteolytic enzymes, Korant was able to dialyze extracts of inhibited infected cells (containing the polyprotein) to remove excess inhibitor and then cleave the polyprotein with other extracts of infected or uninfected cells *in vitro*. The uninfected cell extracts were capable of cleaving the polyprotein to fragments resembling the products of primary cleavage. Secondary cleavages of the polyprotein were mediated only by extracts of infected cells. Korant concluded that the primary cleavages are catalyzed by a host enzyme, while the secondary cleavages are carried out by a different protease which is either virus specified or activated by viral infection.

Similar experiments done with other picornaviruses also indicated that primary cleavages were host-specific while the secondary cleavages were viral-specific. However, each cell line differed with respect to primary cleavage inhibition by TPCK or TLCK, indicating that different cell lines may employ different enzymes in this process (Summers *et al.*, 1972; Lucas-Lenard, 1974; Korant, 1972).

Korant (1973) used iodoacetamide (IAA) as a protease inhibitor and demonstrated the accumulation of the capsid precursor protein. Since this protein was cleaved to capsid-like polypeptides *in vitro* by extracts of infected cells, but not uninfected cells, a viral protease is probably involved in the secondary cleavages.

Zinc ions have also proven to be potent inhibitors of the picornaviral post-translational proteolytic cleavages. Korant et al. (1974) showed that the cleavage of the rhinovirus capsid precursor was most sensitive to zinc. At high concentrations of zinc, precursors with molecular weights higher than the capsid precursor accumulated. Butterworth and Korant (1974) found that with poliovirus and EMC virus the cleavage of the capsid precursor, A, was most susceptible to zinc, but that the cleavage of C → D → E was also sensitive. Korant and Butterworth (1976) proposed that cleavage inhibition was caused by binding of zinc to the viral polypeptides, particularly the capsid precursor. Consistent with this idea was the observation that ^{65}Zn cosedimented with viral capsids in a sucrose gradient. Also, zinc ions prevented the crystallization of HRV-1A virions, and zinc-resistant mutants showed antigenic alterations in their capsid proteins. The cleavage inhibition by zinc ions was reversible since viral precursors labeled in the presence of zinc were properly processed during a chase period in which there were no zinc ions present (Korant and Butterworth, 1976). Nakai and Lucas-Lenard (1976) found inhibition by zinc ions not to be reversible in Mengo virus-infected L-cells. Thus, they concluded that the zinc must complex with the A polypeptide as it is being synthesized on the ribosome such that the resultant change in configuration is permanent.

Amino-terminal amino acid analysis of virion polypeptides has given some insights as to the specificity of the cleavage sites (Matheka and Bachrach, 1975; Ziola and Scraba, 1976; Bachrach et al., 1973; Burrell and Cooper, 1973). In several different picornaviruses aspartic acid or asparagine residues are found in the N-terminal position of VP2,

serine or threonine residues in the N-terminal position of VP3, and glycine in the N-terminal position of VP1. All are breakers of α-helices and initiate β-bends in the folding of protein chains (Lewis and Scheraga, 1971). It has been proposed that the picornavirus precursors are cleaved in β-bends to the left of a helix-breaker amino acid. Carboxyl-terminal analyses of the Mengo virus capsid proteins have been done (Ziola and Scraba, 1976). Leucine, glutamine, glutamine, and alanine were determined to be the C-terminal residues of α (VP1), β (VP2), γ (VP3), and δ (VP4), respectively. Referring to Figure 12, these data indicate that a single viral protease with a specificity for peptide bonds whose carboxyl function is donated by glutamine may be responsible for the conversion of the capsid precursor to ε, α, and γ (Ziola and Scraba, 1976).

This conclusion has recently received support from studies of the translation of EMC viral RNA in a cell-free system. Pelham (1978) has found ε, α, and γ in the protein products of such an in vitro system and has proposed that they are generated from their precursor by the action of a virus specific protease, which is itself a result of primary cleavage.

In the second chapter of this thesis attempts to study the cleavage scheme of Mengo virus proteins in vivo by using the protease inhibitors TLCK, TPCK, PMSF, zinc, iodoacetamide, and carbobenzoxy-L-glutamine chloromethyl ketone (ZGCK) are described.

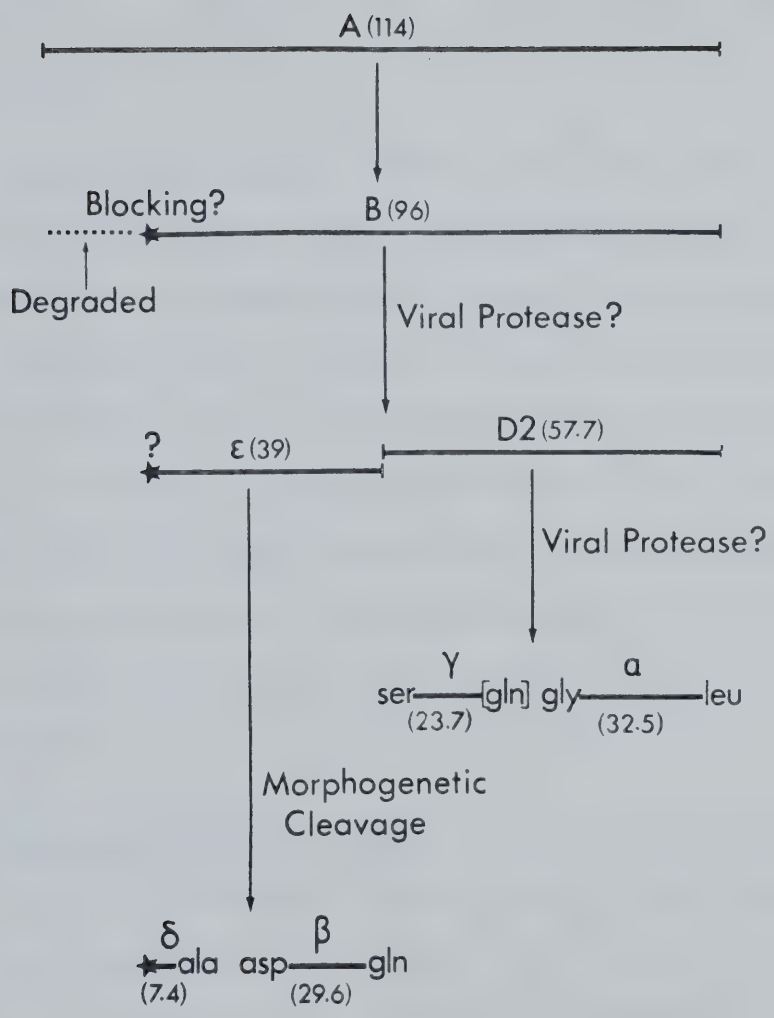


Figure 12. Post-translational processing of the Mengo virus capsid polypeptides. The molecular weights (in thousands) of the polypeptides are shown in brackets.

Materials and Methods

Virus. The M-Mengo plaque variant, originally isolated by Ellem and Colter (1961) was used throughout these studies. The propagation of virus in L-cells, radioactive labeling, and purification of the virus was carried out according to the procedures described by Ziola and Scraba (1974).

Eagle's basal medium. Eagle's basal medium (BME diploid) with Earle's salts and glutamine (catalogue number G-13) was obtained in powder form from the Grand Island Biological Company, Grand Island, New York. It was dissolved in distilled deionized water, and sodium bicarbonate was added to a final concentration of 0.12% before filtration. Before use the BME was supplemented with horse serum (Flow Laboratories, Rockville, Md.) to a final concentration of 5%, penicillin G (Glaxco-Allenburys Ltd., Toronto, Ont.) and streptomycin sulfate (Sigma Chemical Co., St. Louis, Mo.) to final concentrations of 100 IU and 50 µg/ml, respectively.

Amino acid deficient medium. This medium was similar in composition to Eagle's basal medium (diploid) except that it contained twice the normal amount of calcium chloride (i.e., 400 mg/l) and no amino acids other than glutamine. Sodium bicarbonate (final conc.= 0.06%) was added before filtration, after which the sterile medium was supplemented with 1% horse serum and antibiotics as described above.

Virus diluent. The phosphate-buffered saline (PBS) of Dulbecco and Vogt (1954) was supplemented with 0.2% bovine serum albumin, fraction

V (Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y.), 0.002% phenol red (J.T. Baker Chemical Co., Phillipsburg, N.J.) and twice the usual concentration of antibiotics.

Inhibitors. Phenylmethylsulfonylfluoride (PMSF), iodoacetamide, tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK) and tolylsulfonyl lysyl chloromethyl ketone (TLCK) were purchased from Sigma Chemical Company. Zinc chloride was purchased from Mallinckrodt Chemical Works.

Synthesis of carbobenzoxy-L-glutamine chloromethyl ketone (ZGCK). To 80 ml of ether, 20 ml of 40% KOH were added and the mixture was cooled to 5°. To this solution 6.6 g of N-nitroso-N-methylurea were added in small portions with continued cooling and stirring. The deep yellow ether layer was decanted into 20 ml of ether containing KOH pellets and allowed to stand for 3 hr at 0° to remove dissolved water. In another flask 2.5 g of carbobenzoxy-L-glutamine were dissolved in 30 ml of tetrahydrofuran (THF) and chilled to -10° with stirring. Triethylamine (1.4 ml) and ethylchloroformate (0.95 ml) were added and the mixture was left for 9 min at -10°. The diazomethane mixture (40 ml) prepared above was added and after 30 min at 0° the mixture was allowed to warm to room temperature and then extracted in turn with 0.1 M acetic acid and saturated aqueous $NaHCO_3$. The ether layer was dried ($MgSO_4$) and filtered through a "C" sintered disc funnel, then evaporated to dryness on a rotary evaporator and redissolved in 40 ml THF. Five ml of ethanolic 5 M HCl were added, the mixture cooled in ice for 2 min and then extracted in turn with 1 M HCl and saturated aqueous $NaHCO_3$. After drying with $MgSO_4$ and removal of solvent, carbobenzoxy-L-glutamine chloromethyl ketone (ZGCK) was obtained as a crystalline residue,

m.p. 127 °C. A sample was dried for analysis by NMR and by chemical analysis (Found - C, 52.1%; H, 5.4%; N, 8.4%; Cl, 10.4%; O, 23.9%; Calculated for $C_{14}H_{17}ClN_2O_4$ - C, 54%; H, 5%; N, 9%; Cl, 11%; O, 20%).

Infection of monolayers and preparation of cell lysates. Confluent monolayers of L-cells in Petri dishes (approximately 5 x 10^6 cells) were infected at a multiplicity of 100 PFU/cell with Mengo virus suspended in 0.2 ml of virus diluent. After incubation for one hour at 37° to permit attachment of the virus, the monolayers were washed once with warm (37°) PBS and then incubated in 5 ml of BME + 5% horse serum. At 4.5 hr post-infection, the BME + 5% horse serum was replaced by 1.6 ml of amino acid deficient Eagle's medium containing 1% horse serum and 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.0 (D-medium). Fifty min later 0.4 ml of inhibitor (prepared according to figure notes) dissolved in the same medium was added. At 5.5 hr post infection, cells were pulse labeled for 15 min by adding 2 ml of warm (37°) D-medium containing 100 µCi ^{3}H-labeled amino acids/ml (New England Nuclear NET-250) and inhibitor, so that the final concentration of ^{3}H-amino acids was 50 µCi/ml and the concentration of inhibitor was kept constant. To follow the labeling with a chase period, the radioactive medium was removed, the monolayers were washed twice with PBS, and then incubated for 100 min in BME containing 5% horse serum and the same concentration of inhibitor.

Lysates were prepared by removing the medium, washing the monolayers three times with cold (4°) PBS and adding 0.2 ml of "lysis mixture": 0.01 M sodium phosphate buffer, pH 7.2, containing 2% SDS,

5% 2-mercaptoethanol, and 10^{-3} M phenylmethylsulfonylfluoride (PMSF). Lysates were stored at -20° if they were not used immediately.

Preparation of cell extracts. Confluent monolayers in blake bottles (approximately 5 x 10^8 cells) were infected, labeled, and protease inhibitor was added as above except that volumes were 10 times those used in Petri dishes. After the 100 min chase period the cells were rinsed twice with cold (4°) PBS, scraped into a Dounce homogenizer, and broken mechanically with 30 strokes at 4°. They were pelleted at 2000 rpm for 5 min and the supernatent was dialized (if inhibitor had been used) against 1000x volume of 0.002 M phosphate buffer, pH 7.2, with 0.01 M $MgCl_2$, for 24 hr at 4°.

SDS polyacrylamide gel electrophoresis. The procedure used for tube gels has been described (Paucha et al., 1974). Slab gels (1.5 mm thick) contained 5 - 10% acrylamide, 0.2% N,N'-methylenebisacrylamide and 0.1% SDS, in 0.1 M sodium phosphate (pH 7.2). Polymerization was catalyzed by N,N,N',N'-tetramethyl ethylenediamine and ammonium persulfate, at final concentrations of 0.083% and 0.043%, respectively. Samples containing about 120,000 cpm of ^{3}H-labeled viral proteins were made 2% in SDS and 2% in mercaptoethanol and boiled for 10 min. Twenty-five µl of 50% glycerol and 5 µl of bromophenol blue in electrophoresis buffer (0.1 M sodium phosphate, pH 7.2, containing 0.1% SDS) were added to each sample prior to electrophoresis at 35 mA per slab gel. The slab gels were prepared for autoradiography using the method of Bonner and Laskey (1974) as described in the first chapter.

Results

The normal cleavage pattern of Mengo virus polypeptides is shown in Figure 13. At 5 1/2 hours after infection, the cells were pulse labeled for 15 min with radioactive amino acids. A cell lysate was prepared and subjected to SDS polyacrylamide gel electrophoresis as described by Paucha et al. (1974) (Figure 13a). The high molecular weight precursor polypeptides A, B, C, and D were observed in relatively large amounts and the viral polypeptides E, ε, F, α, γ, G, H, and I were also present. If a chase period of 100 min followed the labeling period (Figure 13b), the high molecular weight precursors disappeared and there was an increase in the stable proteins E, ε, F, α, β, and γ.

The effect of TPCK on the processing of Mengo virus precursors is shown in Figure 14. Even at a concentration of 50 μM TPCK there was an accumulation of the capsid precursor polypeptide A. There was also a polypeptide present which is larger than A, having a molecular weight of ∿125,000. There was some inhibition of the D → E cleavage. At 150 μM TPCK the inhibition of cleavage of A was complete, and no capsid polypeptides were seen in the gel. At this concentration of TPCK the conversion of C to D was also inhibited and there was an accumulation of the ∿125,000 molecular weight protein. The presence of this polypeptide indicates that the primary cleavages have been at least partially inhibited. Note also that the stable products of primary cleavages, namely G and H, were absent at this concentration of TPCK.

Figure 15 shows the result of addition of TLCK to the medium of

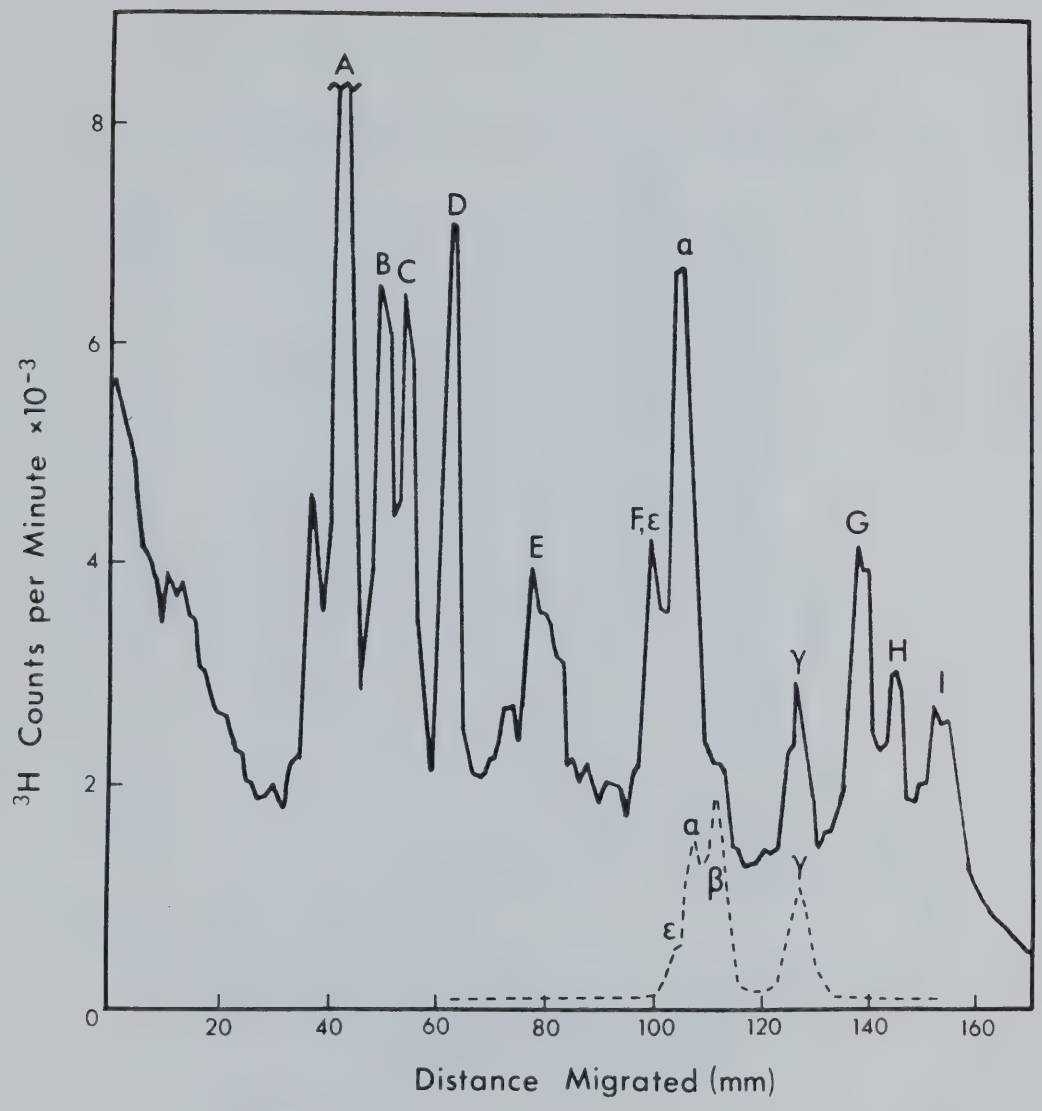


Figure 13a. Polypeptides in L-cells infected with Mengo virus. At 5 1/2 hours after infection cells were pulsed for 15 min with ^{3}H-amino acids, then lysed. Electrophoresis was performed and the tube gels were sliced and assayed for radioactivity using the procedure described by Paucha et al. (1974). ^{14}C-labeled Mengo capsid proteins were included (dotted line) as marker proteins.

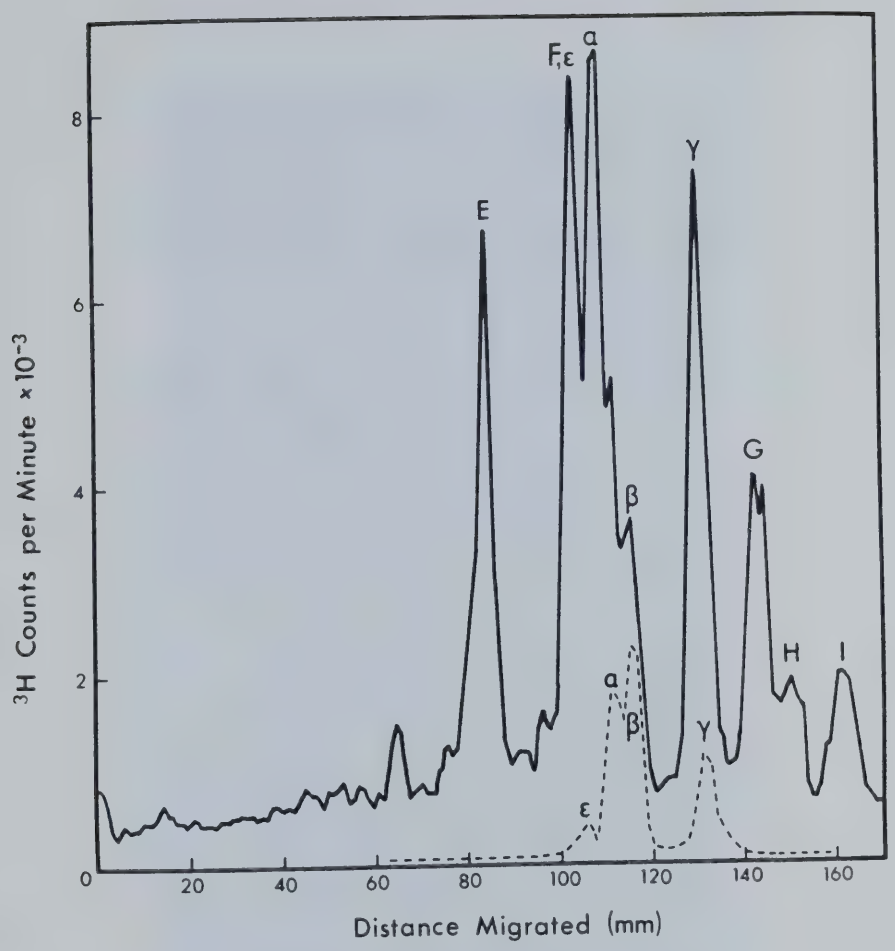


Figure 13b. At 5 1/2 hours after infection cells were pulsed for 15 min in the presence of ^{3}H-amino acids, then incubated for 100 min in the absence of ^{3}H-amino acids before lysis. Electrophoresis was as described by Paucha et al. (1974). The large precursor molecules initially labeled in Figure 13a have been cleaved to generate smaller, stable, viral polypeptides.

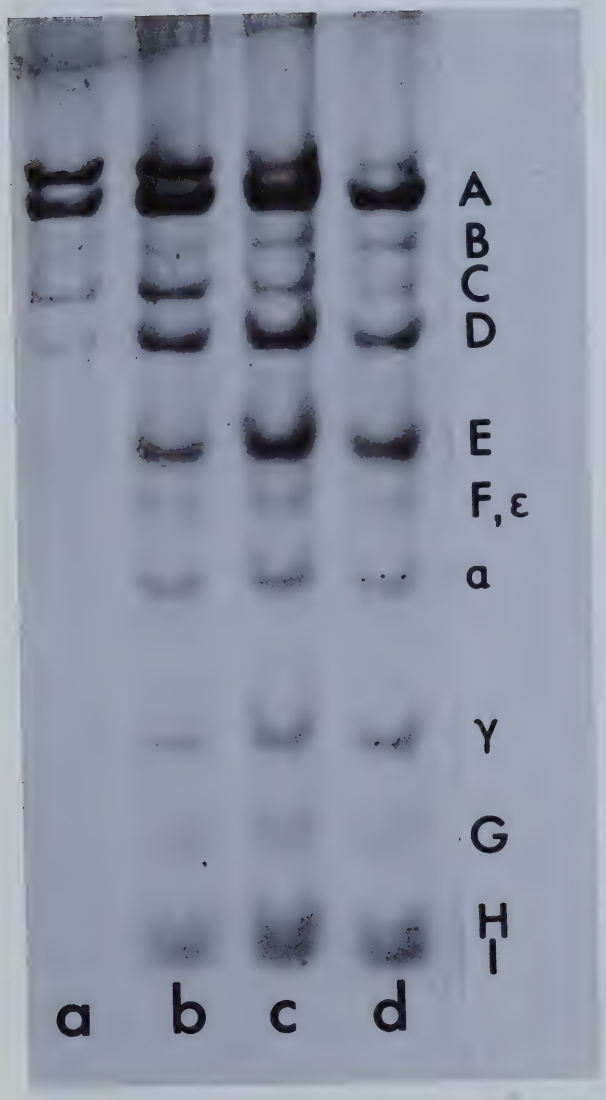


Figure 14. Inhibition of Mengo viral post-translational cleavages by TPCK. TPCK was solubilized in DMSO (10 mg/ml) and added to virus infected L-cells 10 min prior to the addition of radioactive amino acids. The maximum concentration of DMSO was only 1% of the final mixture. The concentration of TPCK was held constant at 150 μM (a), 100 μM (b), 75 μM (c), and 50 μM (d) throughout the pulse and chase periods. Lysates were prepared and electrophoresis in SDS-polyacrylamide slab gels was performed as described in Materials and Methods.

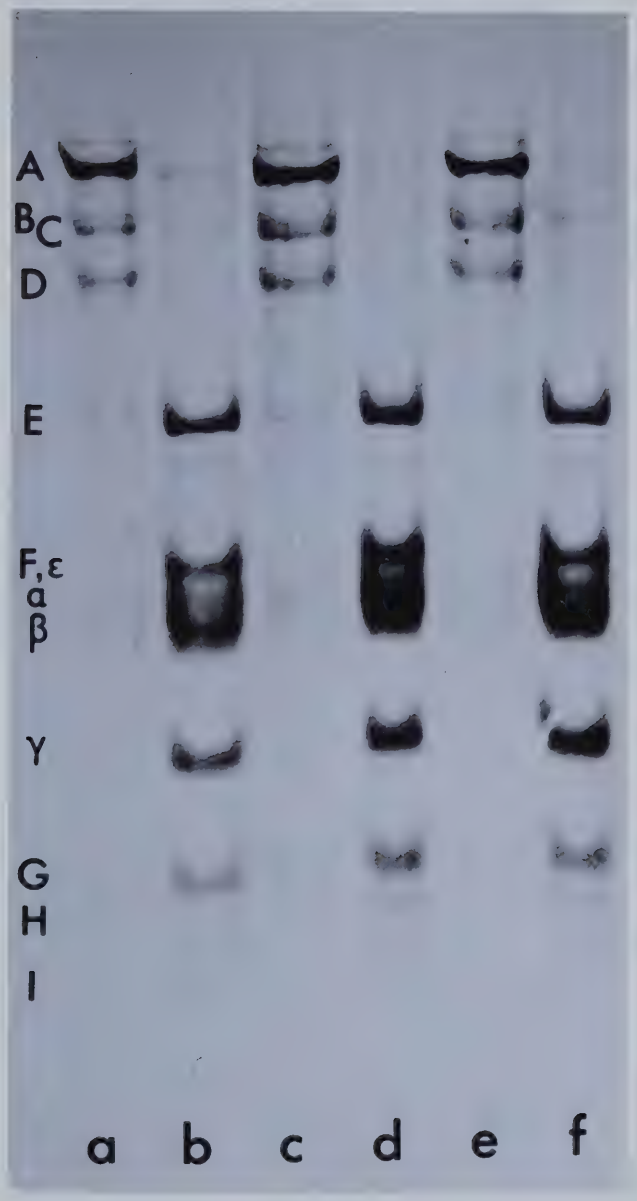


Figure 15. Viral polypeptides observed in Mengo virus infected L-cells treated with TLCK. TLCK was dissolved in DMSO (10 mg/ml) and added to the medium 10 min prior to the addition of radioactive amino acids. Lysates were prepared after a 15 min pulse in the presence of 150 μM TLCK (a) or 75 μM TLCK (c) and also after a 15 min pulse followed by a 100 min chase period (unlabeled amino acids in the medium) in the presence of 150 μM TLCK (b) or 75 μM TLCK (d). Control pulse and chase lysates prepared in the absence of TLCK but in the presence of 1% DMSO are shown in the lanes (e) and (f), respectively.

infected cells. TLCK was found not to inhibit any of the post-translational cleavages of the Mengo viral polypeptides.

The inhibitor ZGCK was synthesized in our laboratory in order to test its effect on the cleavage of viral polypeptides. This compound resembles TPCK or TLCK except that it contains a glutamine moiety and is blocked by a carbobenzoxy group rather than a tosyl group. ZGCK was expected to specifically inhibit proteolytic cleavages in which the carboxyl function of the substrate is donated by a glutamine residue. The results of addition of ZGCK to infected cells are shown in Figure 16. Cleavage of the capsid precursor A was inhibited by the addition of 1 mM ZGCK. At a concentration of 2 mM ZGCK there was nearly complete inhibition of cleavage of A, and the cleavage of C to D and E was also partially inhibited. At this concentration there was an accumulation of two precursor polypeptides with molecular weights of ∿125,000 and ∿135,000. Increasing the ZGCK concentration to 3 mM caused complete inhibition of cleavage of both A and C. The 125,000 and 135,000 molecular weight complexes were also prominent.

The results of inhibition of cleavage of viral polypeptides with iodoacetamide is shown in Figure 17. With this inhibitor there was an accumulation of A beginning at very low concentrations. A corresponding decrease in the amounts of the capsid polypeptides was also seen. The cleavages of C to D and E were seemingly unaffected by iodoacetamide.

Figure 18 indicates the effect of inhibition of the cleavage process by zinc ions. Cleavage of A into capsid polypeptides was partially inhibited at 0.5 to 1.0 mM zinc as was the cleavage of D to E. At higher zinc concentrations, the cleavage of A was more susceptible to

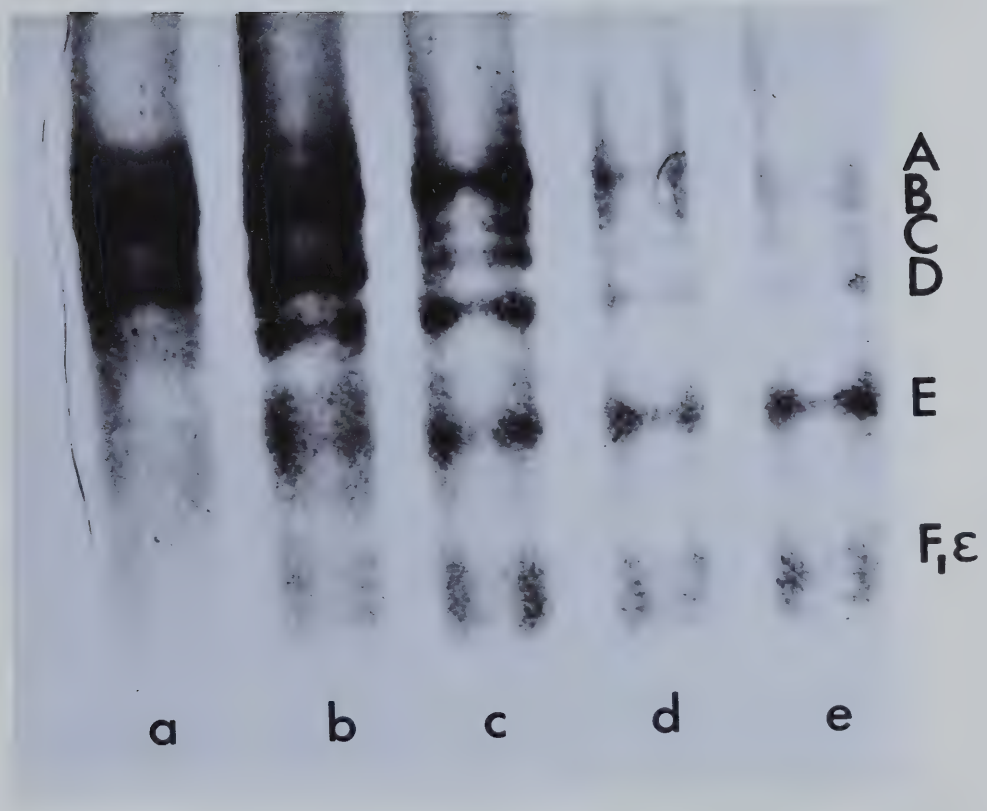


Figure 16. Inhibition of Mengo viral post-translational cleavage by ZGCK. ZGCK was solubilized in DMSO (92 mg/ml) and added to virus infected L-cells 10 min prior to the addition of radioactive amino acids. The concentration of ZGCK was held constant at 3 m$\underline{M}$ (a), 2 m$\underline{M}$ (b), 1 m$\underline{M}$ (c), 0.5 m$\underline{M}$ (d) or 0.1 m$\underline{M}$ (e) throughout the pulse and chase periods. Lysates were prepared and electrophoresed as described in Materials and Methods.

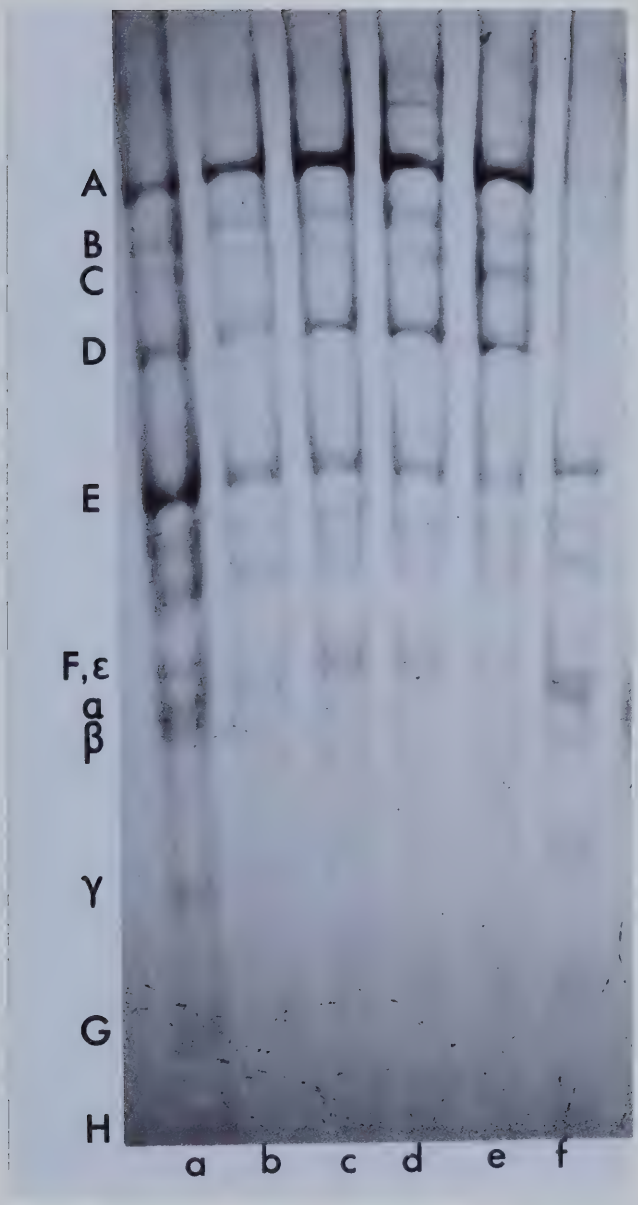


Figure 17. Inhibition of post-translational cleavages by iodoacetamide. IAA was added to Mengo virus infected cells 10 min prior to the addition of radioactive amino acids. The concentration of IAA was held constant at 0.05 m$\underline{M}$ (a), 0.1 m$\underline{M}$ (b), 0.5 m$\underline{M}$ (c), 0.7 m$\underline{M}$ (d), and 1.0 m$\underline{M}$ (e) until after a 100 min chase period. Lysates were prepared and electrophoresed as described in Materials and Methods. A control pulse-chase lysate with no added IAA is shown in (f).

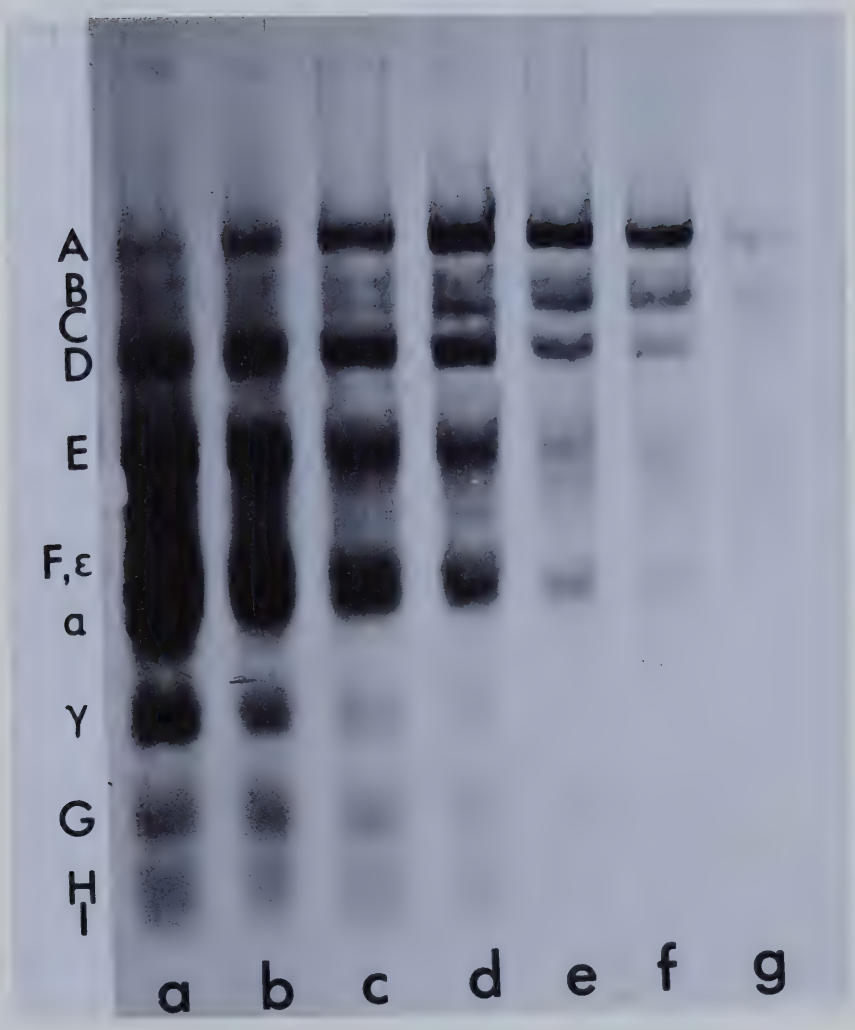


Figure 18. Inhibition of viral polypeptide cleavage by zinc ions. Zinc chloride was dissolved in 1 m$\underline{M}$ HCl and added to infected cells 10 min before the addition of radioactive amino acids. The concentration of zinc was held constant at 0.1 (a), 0.5 (b), 1.0 (c), 1.5 (d), 2.0 (e), 2.5 (f), and 3.0 (g) m$\underline{M}$ until lysates were prepared at the end of the 100 min chase period. Electrophoresis on slab gels was described in Materials and Methods. A control experiment in which a pulse-chase lysate was prepared in the presence of 0.2 m$\underline{M}$ HCl alone showed that HCl had no effect on proteolytic cleavages.

inhibition. In some experiments the two larger molecular weight (125,000 and 135,000) complexes were observed.

PMSF was found to have no effect on the cleavage of Mengo virus polypeptides. This result is shown in Figure 19.

Attempts were made to study the cleavage process of Mengo polypeptides *in vitro*. The cleavage process was inhibited by 0.5 mM iodoacetamide, and labeled cell extracts were prepared according to the procedure described in Materials and Methods. The inhibited extract was then dialyzed to remove IAA, so that the A and D precursors which had accumulated would be susceptible to proteolysis if the appropriate protease were added. Unlabeled extracts of uninfected cells and of cells which were infected but not inhibited were prepared and incubated with the labeled IAA-inhibited extract containing A and D. Electrophoresis was performed, and the results are shown in Figure 20. Neither the infected nor the uninfected cell extracts caused the cleavage of A or D.

Similar results were obtained when labeled extracts prepared in the presence of 1 mM zinc (Figure 21), 1.5 mM ZGCK (Figure 22), and 75 μM TPCK (not illustrated) were mixed with unlabeled extracts from uninfected or infected cells. The failure of extracts to catalyze cleavages of Mengo precursor polypeptides *in vitro* may have been a consequence of conformational alteration in these polypeptides caused by their interaction with the inhibitors.

Discussion

In the presence of low concentrations of TPCK (Figure 14) the Mengo capsid precursor polypeptide A accumulated at the expense of the

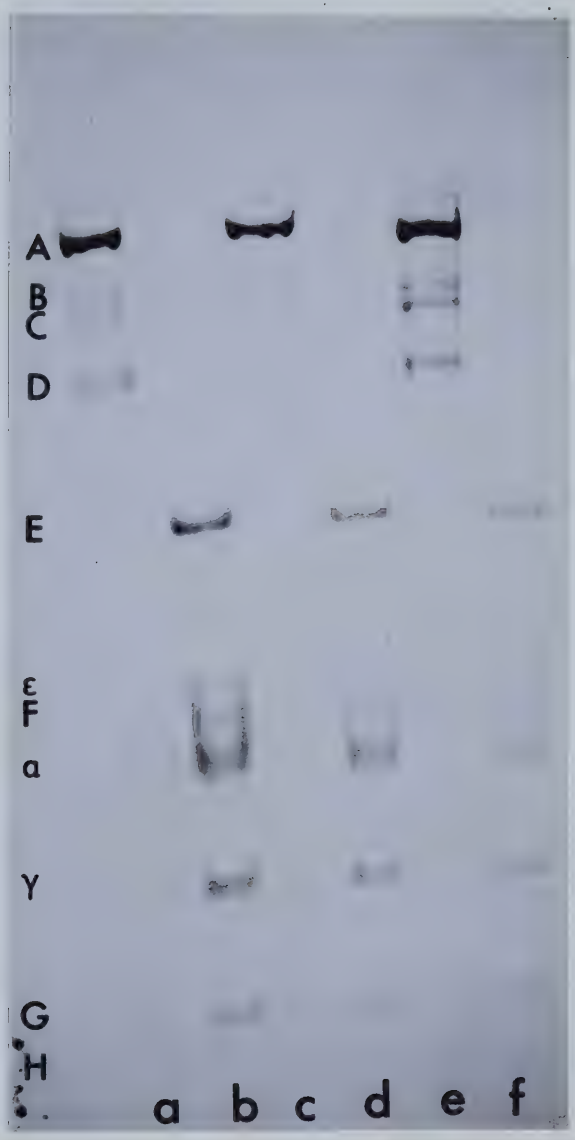


Figure 19. Pattern of polypeptide cleavage observed in Mengo virus-infected cells in the presence of PMSF. PMSF was dissolved in 95% ethanol (0.005 M) and added to infected cells 10 min before the pulse of radioactive amino acids. Lysates were prepared after a 15 min pulse with final concentrations of 1 mM PMSF (a) or 0.5 mM PMSF (c) and also after a 15 min pulse followed by a 100 min chase period with 1 mM PMSF (b) or 0.5 mM PMSF (d). Control pulse and chase lysates prepared in the presence of ethanol are shown in (e) and (f), respectively.

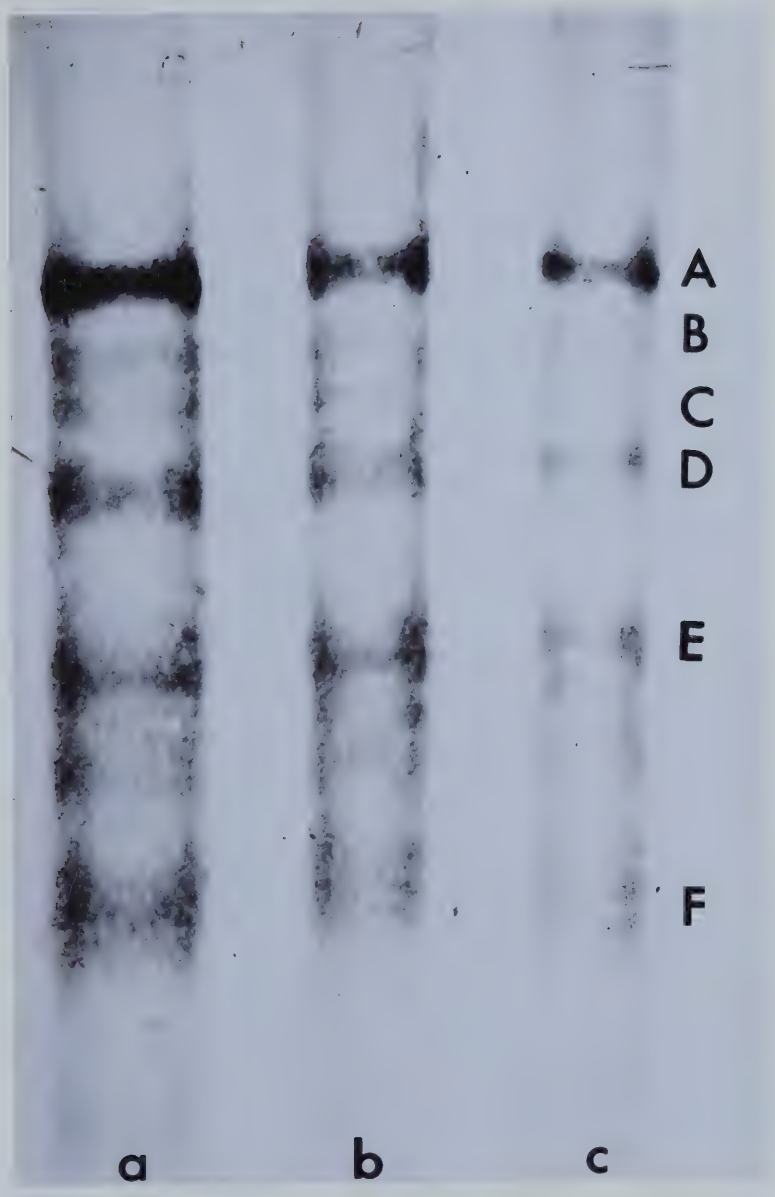


Figure 20. Incubation of labeled IAA-inhibited cell extracts with unlabeled cell extracts. Labeled infected cell extracts were prepared in the presence of 0.5 m$\underline{M}$ IAA (a). After dialysis, incubation with unlabeled infected cell extract (b) or uninfected cell extract (c) was allowed for 3 hr at 37°. Electrophoresis has been described in Materials and Methods.

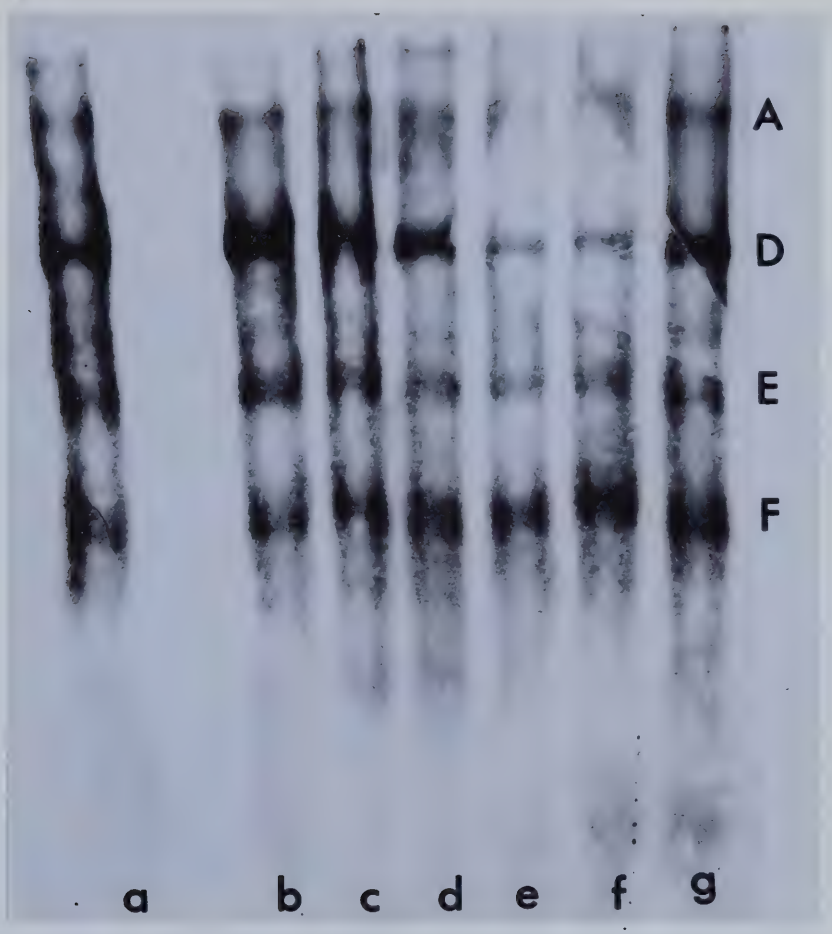


Figure 21. Incubation of labeled zinc-inhibited cell extracts with unlabeled cell extracts. Labeled infected cell extracts were prepared in the presence of 1 mM zinc (a). After dialysis, incubation with unlabeled infected cell extract (b) or uninfected cell extract (c), or with infected cell extracts prepared in the presence of 0.5 mM IAA (d), 1 mM zinc (e), 1.5 mM ZGCK (f), and 75 μM TPCK (g) was allowed for 3 hr at 37°. Electrophoresis has been described in Materials and Methods.

Figure 22. Incubation of labeled ZGCK-inhibited cell extracts with unlabeled cell extracts. Labeled infected cell extracts were prepared in the presence of 1.5 mM ZGCK (g). After dialysis, incubation with unlabeled infected cell extract (a) or uninfected cell extract (b) or infected cell extracts prepared in the presence of 0.5 mM IAA (c), 1 mM zinc (d), 1.5 mM ZGCK (e), and 75 μM TPCK (f) was allowed for 3 hr at 37°. Electrophoresis was described in Materials and Methods.

capsid polypeptides, and cleavage of C → D → E was partially inhibited. At higher concentrations of TPCK, A and a protein that was larger than A accumulated, and there was very little C present. Also, the other products of primary cleavages (namely polypeptides F, G and H) were absent. This would indicate that TPCK inhibited the primary cleavages responsible for producing G, H, F, and C, but did not inhibit the cleavage of the polyprotein to produce the capsid precursor A. The protein larger than A may be part of a C-F-H-G precursor (ΣM.W. for C, F, G and H = 156,000). Because cleavage is inhibited in the presence of TPCK but not in the presence of TLCK (Figure 15), the proteases responsible for primary cleavage probably resembles chymotrypsin in its specificity.

In the presence of ZGCK (Figure 16) the cleavage of A into capsid polypeptides was most sensitive to inhibition. Ziola and Scraba (1976) determined the C-terminal residues of β and γ to both be glutamine, and proposed that the protease resposible for cleaving A into these capsid proteins would have a specificity for bonds in which the carboxyl function of the susceptible peptide bond is part of a glutamine residue. Presumably such a protease would be inhibited by ZGCK, a glutamine analog, resulting in the accumulation of A. ZGCK also caused some inhibition of the cleavage of C → D → E, and two precursors with molecular weights of ∿125,000 and ∿135,000 were accumulated. Thus at the concentration of the inhibitor employed, ZGCK may have acted directly or indirectly as a non-specific protease inhibitor as well as acting specifically on the enzyme responsible for the generation of D2, ε, α, and γ.

At very low concentrations of iodoacetamide A was accumulated at the expense of the capsid polypeptides (Figure 17). Iodoacetamide is

known to bind irreversibly to sulfhydryl groups in proteins, and it is tempting to postulate that the protease responsible for the cleavages of A into capsid proteins contains an essential sulfhydryl group in the enzymatic active site. Alternatively, iodoacetamide may combine with sulfhydryl groups in A and reduce its susceptibility to proteolytic cleavage. Korant (1973) found that the poliovirus capsid precursor polypeptide was accumulated in the presence of iodoacetamide. This precursor could be cleaved to capsid-like polypeptides in vitro by using extracts of infected cells, but not uninfected cells, indicating that a viral specified protease was responsible for this cleavage. This also indicates that the poliovirus capsid precursor accumulated in the presence of iodoacetamide was not altered in its susceptibility to the protease. This protease may specifically cleave bonds in which the carboxyl residue is glutamine.

In the presence of zinc ions the cleavage of A into capsid polypeptides was most sensitive to inhibition (Figure 18). Zinc ions most likely bind to the capsid precursor polypeptide to interfere with its subsequent proteolytic processing (Korant and Butterworth, 1976; Nakai and Lucas-Lenard, 1976).

The experiments which involved adding extracts of virus-infected or uninfected cells to extracts of cleavage-inhibited virus-infected cells were designed to elucidate the roles of cellular vs viral proteases in the cleavage process. However, no proteolysis of Mengo precursor polypeptides was observed in these mixtures in vitro. Perhaps it will be necessary to isolate and purify the A and C precursor polypeptides before assaying their susceptibilities to proteolysis by extracts of virus-infected or uninfected cells.

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